MODULATION OF THE IMMUNE RESPONSE TO FED ANTIGEN IN MICE

Stephan Strobel MD

Thesis presented to the University of Edinburgh for the degree of Doctor of Philosophy in the Faculty of Medicine

1983

EX BIBL. UNIV. EDINBURGEN.

CONTENTS

CHAPTER 2 MATERIALS AND METHODS

CHAPTER 3 NEW TECHNICAL DEVELOPMENTS

CHAPTER 4 ORAL TOLERANCE TO OVALBUMIN AND ITS MODULATION

CHAPTER 4 (Continued)

CHAPTER 4 (Continued)

CHAPTER 5 (Continued)

CHAPTER 6 (Continued)

CHAPTER 7 (Continued)

CHAPTER 7 (Continued)

CHAPTER 8 GENERAL DISCUSSION

Summary

Systemic immunological hyporesponsiveness for humoral and cell mediated immunity (CMI) after enteral antigen exposure (oral tolerance) allows most individuals to ingest foods repeatedly without adverse effects.

Abrogation of this response is considered to be the underlying mechanism in food related hypersensitivities causing intestinal injury and malabsorption. The work of this thesis was designed to investigate, in an animal model, factors which govern the induction and abrogation of oral tolerance and the concomitant expression of intestinal CMI. Feeding ovalbumin to adult mice led to systemic hyporesponsiveness which also prevented the induction of CMI in the gut.

Modulation of the immune response by cyclophosphamide, N-acetyl-muramyl-dipeptide, and a graft-versus-host reaction (GvHR) prior to ovalbumin feeding led to reversal of oral tolerance.

By feeding mice on the first day of life or one day before parturition (by intraamniotic antigen injection), it was demonstrated that early antigen exposure without further immunomodulation primes the animal for systemic immune responses. These findings contrast with the well established ease of tolerance induction in the neonatal period and underline the fact that the route of antigen exposure is of great importance for subsequent immune responses.

The role of the gut was investigated by adoptive serum transfer studies of animals which had been fed ovalbumin. The results show that circulating antigenic moieties are capable of transferring tolerance for

CMI but not for antibodies. Cyclophosphamide pretreatment and X-irradiation of donors abrogated transferable tolerance. Studies of antigen uptake revealed that these effects were not related to quantitative differences in ovalbumin absorption. Further studies were performed to investigate the question whether the abrogation of oral tolerance correlates with the induction of a local CMI on antigen challenge. By histological and morphometric analysis I was able to demonstrate that neonatal antigen exposure and pharmacological immunomodulation prime for local intestinal CMI. In these experiments intraepithelial lymphocyte infiltration has been shown to be an early and reliable marker of CMI in the gut.

I have shown that feeding ovalbumin to neonatal mice does not lead to tolerance but primes for systemic and local immune responses. These animals exhibit signs of an intestinal CMI when the same antigen is introduced into their diets at a later developmental stage. I propose that this effect is due to stimulation of a T-helper/inducer cell population within the gut associated lymphoid tissues (GALT). This phenomenon may underly enteropathies associated with food hypersensitivities in infancy.

Acknowledgements

First and foremost I wish to express my thanks to Dr Anne Ferguson for her continual encouragement, never failing enthusiasm and friendship during the last three years, and for offering me the opportunity to work as a research fellow in the Gastrointestinal Unit of the Western General Hospital.

I thank all my colleagues and friends working in the Wolfson Laboratories and especially those who took it upon themselves to teach, help and advise me on special laboratory methods (haemagglutination assays, enzyme-linked immunosorbent assays): Mrs Margaret Gordon, Mrs Hazel Drummond and Mrs Barbara Walton.

I would like to thank Alexander Sutherland who took care to prepare the histology to a high standard. In addition, I would like to thank Dr Allan Mowat for his friendship, his critical and stimulating discussions and for his help with the early experiments investigating the effects of cyclophosphamide and serum transfer on oral tolerance.

Dr Don Hanson has been a constant source of advice on theoretical matters and played a major part during the development of the anti-ovalbumin enzyme immunoassay.

Miss Maureen A. Pickering and Mr Grant Munro assisted with the animal handling in the serum transfer experiments, and Dr Karl Ziegler helped during the evaluation of intestinal radiation injury. This work would not have been possible without the dedicated help of the Animal Unit, Western General Hospital, and in particular, Miss June Swinton and Mrs Carol Smith.

I am grateful to the Medical Illustration Department, University of Edinburgh and Western General Hospital; to Mr J. Paul for taking photographs of the histology and to Mr G. Pizer and K. Miller for preparing the illustrations.

Miss Carolyn Mailer patiently and skillfully prepared the manuscript.

I am especially thankful to Professor Dr. O. Hövels, Head of the Center of Paediatrics, and Priv. Doz. Dr S. W. Bender, Head, Division of Paediatric Gastroenterology and the staff of the Center of Paediatrics, Frankfurt (F.R.G.) who supported me throughout my research.

I was supported financially in major parts by the Deutsche Forschungsgemeinschaft, by the Medical Research Council of Great Britain, and the University of Edinburgh. I am most grateful to them.

Finally, I would like to express my gratitude to Madeleine, Patrick and Christian for their kindness, encouragement and patience during the preparation of this thesis.

List of publications

The following publications contain major parts of work presented in this thesis:

Mowat, A.McI., Strobel, S., Drummond, H.E., Ferguson, A.
Immunological responses to fed protein antigens in mice.
I. Reversal of oral tolerance to ovalbumin by cyclophosphamide.
Immunology 1982; 45: 105-113

Strobel, S., Mowat, A.McI., Drummond, J.E., Pickering, M.G., Ferguson, A.
Immunology responses to fed protein antigens in mice.
II. Oral tolerance for CMI is due to activation of cyclophosphamide sensitive cells by gut processed antigen.
Immunology 1983; 49: 451-456

Mowat, A.McI., Strobel, S., Pickering, M.G., Drummond, H.E., Ferguson, A.
Evidence that tolerance of cell mediated immunity in mice fed ovalbumin is due to suppressor cells activated by intestinally derived protein moieties.
Ann. N.Y. Acad. Sci. 1983; 409: 853-855

Strobel, S., Ferguson, A.
Immune responses to fed protein antigens in mice. III. Systemic tolerance or priming is related to the age at which antigen is first encountered.
Pediatr. Res. 1983 (in press)

Strobel, S., Ferguson, A.
Oral tolerance - induction and modulation.
Klin. Pädiatr. 1983 (in press)

List of abbreviations

A^{405}	absorbance at 405 nm wavelength
Ab	antibody
B-cell	bone marrow derived lymphocyte
CFA	complete Freund's adjuvant
CMI	cell mediated immunity
CY	cyclophosphamide
ELISA	enzyme-linked immunosorbent assay
F_1	first generation after cross breeding
GALT	gut associated lymphoid tissue
GvHD	graft-versus-host disease
GvHR	graft-versus-host response
hr	hour
I-A	subregion of the murine major histocompatability complex
i.d.	intradermal
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
i.p.	intraperitoneal
irrad-2	two days after irradiation
irrad-5	five days after irradiation
i.v.	intravenous
lyt	T lymphocyte surface antigen (mouse)
MDP	N-acetyl-muramyl-dipeptide
n	number of
OVA	ovalbumin
p	probability
pNS	not significantly different
r	correlation coefficient
R	rad; energy dose of radiation
SAL	saline
SD	standard deviation
SEM	standard error of the mean
T-cell	thymus derived lymphocyte
T_{DTH}	effector T-cell for delayed type hypersensitivity reactions
vs.	versus; compared to
$\bar{x}$	mean

Units

The decimal metre, kilogram, litre system has been used throughout.

Length

nm	nanometre	10^{-9} m
µm	micrometre	10^{-6} m
mm	millimetre	10^{-3} m
cm	centimetre	10^{-2} m
m	metre	1 m

Weight

ng	nanogram	10^{-9} g
µg	microgram	10^{-6} g
mg	milligram	10^{-3} g
g	gram	1 g
kg	kilogram	10^{3} g

Volume

µl	microlitre	10^{-6} l
ml	millilitre	10^{-3} l
l	litre	1 l

Concentration of solutions

M	molar
N	normal

Area

mm^2	square millimetre

Miscellaneous

x g	gravitational acceleration
$\log_{10}$	logarithm to the base 10
pH	negative logarithm to the base 10 of hydrogen ion concentration

Symbols

<	less than
/	per
=	equals

Chapter 1

INTRODUCTION

A. IMMUNOLOGICAL TOLERANCE

Immunological tolerance is defined as a state of specific immunologic hyporesponsiveness to an antigen which, under normal circumstances, would induce an active immune response. The most obvious example is the inability of an organism to be sensitized (immunised) against its own tissue components, although these components are highly immunogenic for other organisms.

The need for the understanding of the mechanisms whereby an antigen can be immunogenic or able to prevent the development of specific cell mediated and/or humoral responses was first stated by Paul Ehrlich in 1904. He tried to explain this central phenomenon by the term "horror autotoxicus", a mechanism by which the organism was prevented from producing antibodies against its own components.

Owen in 1945 observed that dizygotic bovine twins have vascular anastomoses between the two placentae and he found that these twins shared blood group antigens. The erythrocytes however were not being recognised as foreign antigens and those twins possessed a state of acquired immunological tolerance (chimera). This phenomonen was the basis for the theory of tolerance to 'self', originally proposed by Burnet & Fenner (1949). The absence of immune responses to self antigens led them to postulate the existance of a specialised mechanism for the acquisition of self tolerance which was termed "clonal deletion" (Burnet 1959).

A clone is a group of cells all of which are the progeny of a single cell and this theory predicts that the individual carries a

complement of clones of lymphoid cells that are capable of reacting with all possible antigenic determinants. During foetal life or in early stages of their development, clones which react against self antigens are eliminated or killed on contact with antigen thus rendering the organisms specifically unresponsive to self antigens. If different clones came into contact with, for example, soluble or particulate antigens during foetal life, these animals would then be rendered specifically unresponsive to these antigens in later life. Following Burnet's theory, it was shown that dizygotic bovine twins do not reject skin transplants between each other but reject those of other animals (Billingham, Brent & Medawar 1953, Medawar 1961). In pursuing this line, Medawar's group showed that mice of strain A, having been injected with cells of strain B on the 17th day of gestation, do not reject skin transplants of B mice in later life, although they were capable of rejecting skin transplants of other mice. This state of tolerance could be abrogated by injection of lymphoid cells of normal A mice.

Soon afterwards, tolerance to a protein antigen was induced in rabbits (Hanan & Oyama 1954) and further investigations demonstrated that tolerance could be induced to various antigens and that the induction of a specific immunological unresponsiveness is readily achieved in neonatal animals.

It seems that the clonal deletion theory is only one mechanism of central tolerance induction and it is now well recognised that there are multiple ways of tolerance induction which can be summarized as peripheral suppression. These two forms of tolerance are not mutually exclusive and may co-exist. The latter state of tolerance is maintained by active suppression of immunocompetent cells, for example,

T-suppressor cells. Their effects are normally detected as a dampening down of a measurable immune response by regulatory T-lymphocytes which govern the T-T or T-B cell co-operations. The cells and mechanisms involved in the induction of peripheral tolerance will be outlined in the following section.

I. Immune responses and tolerance

The observations of Good & Varco 1955, Miller 1961, Cooper & Ada 1972 and Warner, Szenberg & Burnet 1972, subdividing the immune system into a thymus derived (T) and bursal or bone marrow (B) compartment has been a useful and stimulating concept.

Although additional classes of lymphoid cells including natural killer cells, marrow derived cells, mast cells, dendritic cells and macrophages are all recognised to have important afferent, efferent and regulatory roles in the immune response, I will restrict myself to the discussion of T and B-cells, their ontogeny, interactions, subclasses and their roles in tolerance induction.

1. Ontogeny of B-lymphocytes

Despite the extensive amount of information that has been accumulated on mouse embryology, it has not been possible to identify a tissue equivalent to the bursa in birds and both the gut associated lymphoid tissue and the bone marrow have been proposed as "bursa equivalents". The current view is that B-lymphocytes are derived from hematopoietic stem cells (Wu, Till & Siminovitch 1967) which are themselves orginating from the embryonic yolk sac (Moore & Owen 1967). The yolk sac and the foetal liver seem to be the most likely sites of B-cell differentiation (Phillips &

Melchers 1976) during foetal life. A few days after birth the generation of B-cells in the liver stops (Melchers, von Boehmer & Phillips 1975) and their generation then shifts to the bone marrow (Owen, Raff & Cooper 1975). The earliest recognisable cells of the B-cell lineage are called pre B-cells. They have cytoplasmic IgM, but not surface IgM, and have been described at 11 and 12 days of gestation in the murine foetal liver (Raff, Owen, Cooper, Lawton, Megson & Gathings 1975, Raff, Megson, Owen & Cooper 1976, Melchers 1977). Surface immunoglobulin is first detected at 15 or 16 days of gestation (Nossal & Pike 1973) and at this time is exclusively of the IgM class. IgG and IgA expression occur a few days later. IgD bearing cells appear around 9-10 days after birth. The role or function of surface IgD is not solved and one possible function relates to the regulation of tolerance-susceptibility of immature B-lymphocytes (Vitetta & Uhr 1975). These authors hypothesized that binding antigen by surface IgM alone might deliver a tolerance signal, whereas surface IgD and IgM together might induce differentiation. In support of this theory it has been shown that cells depleted of surface IgD by anti-IgD antibodies were more susceptible to tolerance induction (Scott, Layton & Nossal 1977).

a) Immune responsiveness of murine B-cells

The capacity to mount immune responses against T-dependent and T-independent antigens has been shown to occur in a programmed manner (Sherr, Szewczuk, Cusano, Rapaport & Siskind 1979, Siskind 1981). Murine B-cells are capable of producing an adult like heterogeneous antibody response around 7-10 days of age (Goidl & Siskind 1974). Whether the change in immune responsiveness is due to a differentiation event (Goidl, Klass & Siskind 1976) or under an immunoregulatory influence by T-cells or antigen presenting macrophages has not been resolved. It has been shown, however,

in all transfer studies using syngeneic adult irradiated recipients supplied with an excess of adult T-cells, that B-cell precursors already have acquired the potential to produce IgG antibodies by 14-16 days of gestation (Goidl et al 1976).

The reason for the poor antibody responsiveness to a T-dependent antigen of neonatal mice (Fidler, Chiscon & Golub 1972, Spear & Edelman 1974) cannot be solely attributed to a gross deficiency of T-lymphocytes or immunoglobulin bearing B-cells (Spear, Wang, Rutishauser & Edelman 1973). They demonstrated that adult responsiveness is not achieved before 4-8 weeks of age. Further studies demonstrated that immune responsiveness to T-independent antigens precedes the capacity to respond to T-dependent antigens during ontogeny (Rabinowitz 1976, Hardy & Mozes 1978).

The suppression of antibody synthesis of neonatal mice was shown by Mosier and Johnson (1975) to be associated with an excess of endogenous, non-specific suppressor T-cells which interfered with the function of T-helper cells. They demonstrated that neonatal thymocytes constitute a rich source of T-suppressor cells for adult antibody synthesis (Mosier, Mathieson & Campbell 1977) emphasising the close immunoregulatory links between and B and T-cells.

2. Ontogeny of T-lymphocytes

The T-cell system has two primary roles. The first is regulation of the immune response through antigen recognition and co-operation with other immunocompetent cells.

The second role is the effector function of T-cells. They are responsible for cytotoxicity and delayed type hypersensitivity reactions as well as for suppressing the development of autoimmune phenomena.

T-cells orginate from the foetal yolk sac, liver or adult bone marrow-derived hemotopoietic stem cells which migrate to the thymus (Owen & Raff 1970, Stutman & Good 1971). This migration is an absolute requirement for further differentiation of T-lymphocytes in vivo (Stutman 1978).

After the demonstration of a subpopulation of immunologically competent T-cells within the thymus (Raff 1971), the model of T-cell differentiation was based on the following sequence: hematopoetic stem cell - (thymus) thymocyte - mature thymus lymphocyte - peripheral T-lymphocyte (Raff 1971,1973).

Several theories have been proposed to explain the development of subsets of mature T-cells each of which have different functional characteristics. The main differences between the different theories proposed by Raff (1971,1973) and Stutman (1978,1981) are related to the influence of antigens on T-cell maturation, and to the restriction by the major histocompatibility complex. T-cells may require antigen exposure before they differentiate into functionally different populations (Raff 1971,1973) or they may even be precommitted to a certain activity before antigen exposure (Stutman 1981).

a) T-lymphocyte cell surface marker

The murine thymus contains thymocyte precursor cells as early as 12-13 days of gestation (Chakavarty 1977) and by 19 days of gestation the expression of Thy 1+, Lyt 1+, Lyt 2+ antigens reach adult levels (Kamarck & Gottlieb, 1977).

The expression of several murine membrane antigens is linked with maturational events within the thymus and with peripheral immunoregulatory function. Thy 1+ antigen is mainly found on prethymocytes and is reduced in quantity during development. Lyt antigens are developed during maturation

and cells which undergo further functional differentiation will lose either the Lyt 1+ or Lyt 2,3+ marker (Cantor & Boyse 1975,1977). When T-cells are released into the periphery from the thymus they contain roughly 50% Lyt 1,2,3+, 30% Lyt 1+,2- and less than 10% Lyt 1-;2,3+ cells. By using different subsets of specific antisera and complement, the following functions can be associated with the membrane markers listed below:

Lyt 1,2,3+ : amplifier cell, nonspecific suppressor cell

Lyt 1+;2,3- : helper/inducer cell, probably also DTH effector cell

Lyt 1-;2,3+ : specific suppressor cell; cytotoxic cell

(Gershon 1975, Feldman, Beverley, Dunkley et al 1977, Waksman 1977).

Except for the cytotoxic or natural killer cells, activated T-cells exert their influence by the production of soluble, antigen specific T-cell factors, lymphokines. These factors are mediator molecules and perform the function of those cells in an antigen specific manner and thus play an important part in immunoregulation (Tada & Okumura 1979, Germain & Benacerraf 1980). Whereas antigen specific suppressor factors have been demonstrated in many experimental conditions for antibodies (Feldman 1974, Tada, Taniguchi & Takemori 1975, Kontiainen, Howie, Maurer & Feldman 1979) and for delayed type hypersensitivity reactions (Kojima, Tamura & Egashira 1979, Liew, Sia, Parish & McKenzie 1980), soluble helper factors for delayed type hypersensitivity have so far not been demonstrated.

The important and crucial role of the T-cell in tolerance induction, modulation and maintenance will be discussed in the following section.

II. Mechanisms operative in immunological tolerance

It has become more and more evident that immunological tolerance cannot only be attributed to one central mechanism (clonal deletion) but that it is the outcome of a delicate network of different immunoregulatory control mechanisms. These mechanisms are not mutually exclusive and can co-exist in the same animal (Waksman 1977). In this section I shall confine myself mainly to the discussion of tolerance to protein antigens and describe the mechanisms for parenteral induction of T and B-cell tolerance.

The mechanisms of tolerance can be broadly distinguished by their mode of action and described as direct (passive) leading to removal of B-cell clones from reactivity (reviewed by Nossal & Pike 1980) or as indirect (active) by activation of specific suppressor T-cells which then act on T or B-cells (Gershon & Kondo 1970,1971, Waksman 1977). The mechanisms responsible differ according to the various experimental systems.

Specific tolerance can be induced by a wide range of antigens and a variety of experimental in vivo and in vitro conditions and results in unresponsiveness of all limbs of the immune system.

Reversible receptor blockade or functional deletion of B-cell has been demonstrated by Aldo-Benson & Borel(1974) in experiments coupling hapten conjugates to isologous gamma globulin and by Howard & Mitchison (1975) using pneumococcal polysaccharides. Clonal deletion of B-cells in vitro by using lipopolysaccharides and antigen antibody complexes has been demonstrated by Diener & Feldman (1972). Clonal deletion during perinatal tolerance induction and clonal abortion in adult

animals has been demonstrated also by Nossal and Pike (1975,1980).

The relevance of these mechanisms for tolerance at the T-cell level is still unproven, although specific T-cell deletion is known to occur (Cooper & Ada 1972, Godfrey 1976). The balance between tolerance and sensitisation is delicate depending on antigenic structure, dose, immune status of the animal and route of antigen presentation. It was shown that unusually high or low doses are tolerogenic on intravenous injection (Dresser 1962, Dresser & Mitchison 1968) and experiments by Chiller, Habicht & Weigle (1971), Weigle (1977) and Parks & Weigle (1980a) demonstrate that deaggregated human gamma globulin is tolerogenic whereas aggregated human gamma globulin is an obligate immunogen. Generally, this phenomenon seems to be dependent on the epitope density of antigens. Low epitope density renders antigens rather tolerogenic whereas antigens with a high epitope density are more likely to immunise.

Antigen presentation to T-cells directly or via macrophages has been shown to activate a certain population of these cells and to create a state of tolerance transferable by spleen cells. Tolerance for CMI in mice seems to be very sensitively controlled by T-suppressor cells as has been shown in studies by various groups using particulate and skin sensitising antigens (Basten, Miller, Sprent & Cheers 1974, Claman, Phanuphak & Moorhead 1974, Kaufman & Hahn 1979, Asherson & Zembala 1980). Whether the tolerant state is maintained by active suppression appears to be dependent on the experimental system. In this respect the absence of demonstrable T-suppressor cells in tolerant animals (Parks, Doyle & Weigle 1978, Parks and Weigle, 1980b, Hanson & Miller 1982) strengthens the possibility that more than one mechanism may be

responsible for maintaining tolerance. On the other hand, a deficiency of T-helper cells is responsible for immunologic hyporesponsiveness after intravenous injection of ovalbumin (Endres & Grey 1980a,b). Furthermore, the hyporesponsive state can also be induced by immunoregulatory feedback suppression. This immunoregulatory circuit depends on activation of T-helper inducer cells (Lyt 1+) which are directly stimulated by antigen to become inducers of T-suppressor cells (Lyt 2,3+) which will then at the end of the circuit suppress Lyt 1+ helper cells (Eardley, Hugenberger, McVay-Boudreau, Shen, Gershon & Cantor 1978, Shimamura, Hashimoto & Sasaki 1982, McDonald 1982). This circuit can also be triggered off by immune B-cells (Shimamura et al 1982).

1. Regulation of cell mediated immunity (CMI)

In general, humoral and cell mediated immune reactions are both dependent on the balance between systemic sensitisation or suppression and in this section I will discuss the regulation of CMI or delayed type hypersensitivity reactions. The term 'cell mediated immunity' encompasses various forms of immune reactions involving T-cells, macrophages, basophils, eosinophils or polymorphonuclear cells (Waksman 1979). The classical CMI reaction (tuberculin type) is T-cell mediated. $T\text{-}_{DTH}$ cells release lymphokines, for example, a macrophage activation factor, and produce a macrophage dominated inflammatory lesion. Suppressor T-cells appear to play a major part in regulating the induction and expression of CMI (Röllinghoff, Starzinski-Powitz, Pfizenmaier & Wagner 1977, Gill & Liew 1978, L'age- Stehr & Diamantstein 1978) whereas the existence of soluble helper factors for CMI have still not convincingly been demonstrated (see review Taussig 1980).

The induction of T-helper and $T\text{-}_{DTH}$ effector cells show differences in their dose responses to the same antigen. T-cell stimulation usually requires antigen presentation on the surface of a macrophage. T-cell subsets, T-helper or $T\text{-}_{DTH}$ cells which are Lyt 1+ but not Lyt 2,3+ in mice are triggered only when they recognise I-region associated (Ia) antigens of the macrophage surface in close connection with the antigen; a process which is called 'dual recognition' (Bretscher & Cohn 1970). The activation of antigen-specific suppressor T-cells of Lyt 2,3+ phenotype is restricted commonly by K or D region antigens of the major histocompatibility complex (see review Zinkernagel & Doherty 1977). Since T-helper and $T\text{-}_{DTH}$ cells share the same surface markers and major histocompatibility complex restricted recognition pathway (Miller, Vadas, Whitelaw & Gamble 1976) it remains to be proven whether these are functions of T-cells of different origin rather than alternative operative or developmental functions of the same cell.

B. STRUCTURE AND DEVELOPMENT OF THE GUT ASSOCIATED LYMPHOID TISSUES (GALT)

There is now ample evidence that antigen within the lumen of the gut can trigger off an immune response which may manifest both locally in the lamina propria and/or systemically throughout the body. Because of the unique exposure of the gastrointestinal tract to a wide range of complex antigenic stimuli, a complete range of different defence and effector mechanisms are found in the epithelium and lamina propria of the gut mucosa. These include mainly IgA and, possibly, IgM secreting plasma cells, effector T-cells including suppressor, helper and killer cells as well as macrophages, monocytes, eosinophils and mast cells (see

review Parrott 1976, Waksman & Ozer 1976, Bienenstock & Befus 1980).

I shall first deal with special features of the gut associated lymphoid tissue (GALT), migration pathways of T and B blasts and discuss the heterogeneity of intraepithelial lymphocytes and their possible relationships to cells of T and/or mast cell lineage.

Since the recognition that IgA is the main secretory immunoglobulin (Tomasi, Tan, Solomon & Prendergast 1965), it has been shown that the Peyer's patch is the main source of IgA committed blast cells. The Peyer's patches are lymphoid aggregates within the intestinal mucosa of the gut. The nodular area of the lymphoid follicles is a B-cell area whereas the T-cell area is a small and narrow internodular zone. It contains also the post-capillary venules with their highly specialised epithelium which plays an important role in lymphocyte traffic (Parrott & de Sousa 1971). The epithelium over the dome area is cuboidal instead of columnar and lacks goblet cells. Work on human Peyer's patches (Owen & Jones 1974) and in mice (Owen 1977) has shown that there is a specialised M (microfold) cell which seems to be a major route for intraluminal antigen access and presentation to immunocompetent cells in the Peyer's patch (Owen 1977).

In the mouse, Peyer's patches can be identified before birth as aggregates of reticular cells, and these areas are populated by lymphocytes as early as three days after birth (Ferguson & Parrott 1972). Primary nodules appear around 3-7 days and germinal centres at 4-5 weeks of age (Ferguson & Parrott 1972, Waksman 1973).

I. Lymphocyte traffic

Gowans and Knight (1964) first demonstrated in rats that thoracic duct lymphocytes have a selective tendency to 'home' into lymphoid

tissues including the Peyer's patch (small lymphocytes) and that labelled large lymphocytes migrate from the blood into the gut wall where they assumed the appearance of plasma cells. This work has been confirmed and extended (Hall & Smith 1970, Parrott & de Sousa 1971, Guy-Grand, Griscelli & Vassalli 1974). In the Peyer's patch it has been shown that thymus cells are confined to T-dependent areas between the nodules.

There is still some debate whether there exists a common mucosal associated lymphoid system with free cell traffic or whether there is only a restricted traffic between different mucosal sites, for example, between the gut associated and the bronchus associated lymphoid tissues. In a rabbit model, Rudzik, Clancy, Perey & Bienenstock (1975) demonstrated that IgA containing cells from the gut associated lymphoid tissue, had a propensity to repopulate the bronchial mucosa. Goldblum, Ahlsted & Carlsson (1975) and Hanson, Carlsson, Cruz, Dahlgren, Garcia & Urruttia (1981) demonstrated an enteromammaric axis when they showed that antigenic exposure of the intestine resulted in secretory antibodies appearing in breast milk, probably due to the traffic of Peyer's patch cells to the mammary gland.

Investigations of the traffic/homing pattern demonstrated that immunoblasts begin to enter the gut within two hours after injection and reach a maximum after 24 hours (Hall & Smith 1970) and that the differentiation from blast cells to immunoglobulin producing plasma cells takes place within the lamina propria (Hall, Parry & Smith 1972, Parrott & Ferguson 1974, Guy-Grand et al 1974). The factors controlling lymphoblast localisation in mucosal tissues are still not completely understood. In experiments in which hypothetical receptors responsible for the localisation of IgA-lymphoblasts were blocked, it

was shown that their localisation was not dependent on the 'secretory piece' or immunoglobulin receptors (McWilliams, Phillips-Quagliata & Lamm 1975). Factors such as antigen exposure (Husband & Gowans 1978), hormones (Weisz-Carrington, Roux, McWilliams, Phillips-Quagliata & Lamm 1978) and blood supply (Ottaway & Parrott 1980) all have been shown to influence the 'homing' pattern of lymphoblasts to secretory sites. T-blasts from the thoracic duct and mesenteric lymph nodes seem to have an antigen independent homing pattern as they lodge selectively in the lamina propria and epithelium of both normal small intestine and of antigen free foetal gut isografts implanted under the kidney capsule (Parrott & Ferguson 1974, Guy-Grand et al 1974, Sprent 1976).

Peripheral T-blasts do not localise in the intestine unless the mucosa is inflammed (Rose, Parrott & Bruce 1976).

1. Memory of gut associated lymphoid tissues

It is well known that further antigen administration after primary immunisation can lead to an anamnestic, secondary, response which takes a shorter time to reach the maximum, affects mainly the IgG system and is usually higher than the primary response. The question whether the intestinal immune system has a classic immunological memory is still a matter of some debate and seems to be dependent on the experimental conditions and antigens.

Experiments in which counts were performed of cells synthesing IgA in the thoracic duct and lamina propria after a parenteral immunisation, followed by an oral challenge, demonstrated an anamnestic response (Pierce and Gowans 1975, Husband and Gowans 1978). However, measurements of specific antibodies in intestinal secretions did not show classical memory in dogs (Pierce, Sack & Sircar 1977). Injection

of bacteria into rat Peyer's patches (Andrew & Hall 1982), however, produced clear evidence of a secondary IgA response in the bile. Whether this is the case after oral antigen administration remains to be established.

II. Mucosal and intraepithelial lymphocytes and local cell mediated immunity

Lymphocytes are found throughout the gastrointestinal tract and are numerous in the organised lymphoid tissues such as Peyer's patches and mesenteric lymph nodes. In the mucosa they are found in the lamina propria and within the epithelium.

An increase of intraepithelial lymphocytes during a local CMI (Ferguson 1973) or a reduction in antigen deprived small intestine or in thymectomized mice (Ferguson & Parrott 1972) indicate an important immunological role of these cells.

Intraepithelial cells were first described almost 100 years ago (cited by Stenqvist 1934) and studied in more detail by Fichtelius, Yunis & Good (1968), Ferguson and Murray (1971), Ferguson (1973, 1977) and Otto (1973). Intraepithelial lymphocytes constitute about 10% of the epithelial cells in mice. In guinea pigs they have been shown to respond to cell mitogens and to exhibit antibody dependent cell mediated cytotoxity in vitro (Arnaud-Battandier, Bundy, O'Neill, Bienenstock & Nelson 1978). Similar functions have been ascribed to lamina propria lymphocytes which have been shown to respond to phytohaemagglutinin (PHA) and concanavalin A (Con A) and in mixed lymphocyte reactions (Singal, O'Neill, Clancy & Bienenstock 1976,

Arnaud-Battandier et al 1978, Arnaud-Battandier, Wahl & Nelson 1979).

Intraepithelial lymphocytes are not a homogeneous population. They appear to consist of small and large cells (Fichtelius, Finstad & Good 1969, Glaister 1973). The number changes appreciably with age in mice, adult levels being reached three weeks after birth around weaning. Cell surface marker studies (Selby, Janossy, Goldstein & Jewell 1981, Lyscom & Brueton 1982, Schrader, Scollay & Battye 1983) of isolated, intraepithelial and mucosal lymphocytes preparations revealed that the majority (around 70%) are of the suppressor/cytotoxic phenotype Lyt 2,3+, whereas the rest are of Lyt 1+ phenotype (helper). The majority of intraepithelial cells are of lymphoid character by morphological criteria and several workers have described granules similar to those of mast cells within the cytoplasm of these cells. These granules however are smaller and fewer than those found in connective tissue mast cells (Murray, Miller & Jarrett 1968, Rudzik & Bienenstock 1974).

Intestinal mast cells are found in substantial numbers in the lamina propria of rats. They are situated mainly around the crypts and differ from connective tissue mast cells in their morphological (Enerbäck 1966a) and histochemical staining properties (Enerbäck 1966b).

Intraepithelial (atypical) mast cell infiltration is part of an important protective host response against nematode infections. Worm expulsion in rats actively immunised with Nippostrongylus brasiliensis is clearly related to an infiltration with atypical mast cells or globule leucocytes (Miller & Jarrett 1971). This response seems to be under control of the thymus (Olson & Levy 1976, Ruitenberg & Elgersma

1976). The population of these cells is also expanded during a GvHR (Guy-Grand et al 1978, Mowat & Ferguson 1982) and thought to be a measure of gut cell mediated immune responses during the GvHR. Although T-cell markers have been identified on these cells (Guy-Grand et al 1978, Schrader et al 1983) a sharp distinction between mucosal mast cells, intraepithelial granulated cells and intraepithelial lymphocytes has not yet been drawn. It seems likely that intra-epithelial granulated cells and mucosal mast cells are of different lineage. Recent work (Kitamura, Shimada, Hatanaka & Miyano 1977, Mayrhofer 1980, Kitamura, Yokoyama, Matsuda, Ohno & Mori 1981) has shown that mast cells are probably of (hematopoietic) bone marrow origin contrasting with the theory of Burnet (1977). The sparsely granulated cells are not a homogeneous population and may account for natural killer, suppressor and cytoxic activity seen in gut mucosal and intraepithelial lymphocyte preparations (Davies & Parrott 1981, Schrader et al 1983, Parrott, Tait, Mackenzie, Mowat, Davies & Micklem 1983). The T-cell dependency of experimental villus atrophy in the small intestine of rats during a mematode infection (Ferguson & Jarrett 1975) is evidence of the important immunoregulatory role of the T-cell system. It could be taken as indirect evidence that mucosal T-cells - necessary for a local CMI to develop - also have effector functions. Athymic mice, for example, are deficient in clearing a usually self-limited giardia infection (Roberts-Thomson & Mitchell 1978).

An increase of intraepithelial lymphocytes during an immunologically mediated mucosal damage in mice has been demonstrated during a GvHR (Guy-Grand et al 1978), allograft rejection (MacDonald & Ferguson 1976) and during a local CMI against ovalbumin in mice (Mowat

& Ferguson 1981). In man, a raised intraepithelial lymphocyte count is found in cows' milk protein intolerance and coeliac disease (Ferguson 1974, Mavromichalis, Brueton, McNeish & Anderson 1976, Phillips, Rice, France & Walker-Smith 1979). Whether these cells have an effector or mainly an immunoregulatory role or are only secondarily recruited to the site of tissue damage remains to be established.

C. IMMUNE RESPONSES AFTER ENTERAL ANTIGEN PRESENTATION

ORAL TOLERANCE

The phenomenon of hyporeactivity after enteral antigen administration was noted by Dakin (1829) who described the practice of American Indians of ingesting leaves of the poison ivy plant to prevent or alleviate symptoms which occurred after a cutaneous encounter. Orally or parenterally administered poison ivy extracts or a combination of both have been used to prevent or alleviate symptoms due to contact allergy for a long time (Stevens 1945).

When the first encounter with an antigen is by the enteral route, a local secretory IgA antibody response will ensue (Heremans 1974). Small amounts of protein antigens are absorbed intact (Uhlenhuth 1900, Lippard, Schloss & Johnson 1936, Swarbrick 1979, Walker 1981) and thus may induce systemic humoral immune responses (Gruskay & Cooke 1955, Rothberg & Farr 1965, Rothberg, Rieger, Silverman & Peri 1981) or systemic CMI which has been demonstrated by Perrotto, Hang, Isselbacher & Warren (1974) after feeding a soluble egg antigen of Schistosoma mansoni.

It is important to note however that circulating food related antibodies are not synonomous with disease and are frequently demonstrated in healthy individuals.

It was shown in the early experiments of Wells and Osborne (1911) that oral administration of antigen is extremely effective in inducing systemic tolerance. He demonstrated that the anaphylatic response of guinea pigs towards egg white or corn protein could be prevented by prior feeding of the antigen in question. This was confirmed for contact sensitising agents (Chase 1946, Asherson, Zembala, Perera, Mayhew & Thomas 1977) heterologous red cells (André, Heremans, Vaerman & Cambiaso 1975), Kagnoff 1978a,b) and for proteins (Thomas & Parrott 1974, Hanson, Vaz, Maia, Hornbrook, Lynch & Roy 1977, Ngan & Kind 1978, Miller & Hanson 1979).

Under normal conditions, both limbs of the immune response are rendered unresponsive after an oral feed. Suppressed total serum antibody responses (Hanson et al 1977, Kagnoff 1978a) specific IgE responses (Ngan & Kind 1978, Vaz, Maia, Hanson & Lynch 1977) and CMI responses (Chase 1946, Asherson et al 1977, Kagnoff 1978a, Miller & Hanson 1979) have been reported to be suppressed after antigen feeding.

The underlying mechanisms for the unresponsive state are still unclear and both cellular and humoral mechanisms have been implicated. It was found that serum from orally immunised mice was capable of suppressing the immune response <u>in vivo</u> and <u>in vitro</u> (André et al 1975, Kagnoff 1978a, Chalon, Milne & Vaerman 1979). Mechanisms held responsible for the suppression of humoral immune responses after feeding sheep erythrocytes include circulating antigen-antibody immune complexes (André et al 1975) and - supported by better evidence -

anti-idiotypic antibodies (Kagnoff 1980). T-suppressor cells were however required to suppress delayed type hypersensitivity reactions (Kagnoff 1980). These cells have been shown to be generated in rat Peyer's patches after feeding sheep erythrocytes (Mattingly & Waksman 1978).

To date, no circulating suppressive serum factors could be demonstrated after feeding soluble antigens and induction of T-suppressor cells seems to be the prevalent mechanism for inducing hyporesponsiveness to protein antigens (Thomas & Parrott 1974, Ngan & Kind 1978, Richman, Chiller, Brown, Hanson & Vaz 1978, Miller & Hanson 1979) and skin sensitising agents (Asherson et al 1977).

The liver plays a role in tolerance induction (Triger, Cynamon & Wright 1973, Thomas, Ryan, Benjamin, Blumgart & MacSween 1976) by filtering out antigenic moities as demonstrated by the abrogation of tolerance after portocaval shunting in dogs (Cantor & Dumont 1967). Much further work to elucidate the role of the liver is needed; and whether the abrogation is due to circumvention of specialised liver macrophages remains to be established (Richman, Klingenstein, Richman, Strober & Berzofsky 1979).

I. Immunoregulation by class specific regulatory T-lymphocytes

A hypothesis for immunoregulation of systemic or local immune responses after oral antigen exposure has been proposed recently (Elson, Reilly & Rosenberg 1977, Strober, Richman & Elson 1981) and implies a crucial immunoregulatory role for the Peyer's patch. Antigenic stimulation within the gut associated lymphoid tissues

induces T-helper and T-supressor cells which may account for systemic unresponsiveness and local mucosal responsiveness following antigen feeding.

A class specific regulation of mucosal immune responses has been proposed, local mucosal IgA responses being induced after feeding and systemic IgG and IgM responses being suppressed. These experiments and theories still leave many aspects unexplained, for example, how a mucosal IgM response can be induced by feeding.

D. MODULATION OF IMMUNOLOGICAL TOLERANCE

One of the enigmas in immunology is that the immunological response obtained depends critically on the route of antigen administration. This is very well demonstrated in the case of contact sensitivity where intravenous injection (Claman 1979) and prior feeding of a contact sensitising agent (Asherson et al 1977) induce a state of tolerance, whereas topical application leads to contact sensitivity (Asherson et al 1977, Claman, Miller & Moorhead 1977). In general, intravenous injections of high and exceptionally low antigen doses lead to tolerance, whereas intermediate range doses lead to immunisation (Dresser & Mitchison 1968). The dose of antigen has also been shown to be important for induction of T and/or B-cell tolerance (Chiller & Weigle 1971, Weigle 1977).

Furthermore, the antigenic structure (epitope density) has been shown to be equally important since deaggregation of human gamma globulin (Chiller and Weigle, 1971) renders it an obligate tolerogen, whereas aggregated human gamma globulin is immunogenic.

It was further shown that *in vitro* digestion (Dosa, Pesce, Ford, Muckerheide & Michael 1979) generated tolerogenic and immunogenic fragments. It is assumed that the underlying mechanism depends on the capacity of the immunoregulatory cells to recognise the antigen as tolerogenic without macrophage presentation or as immunogenic when presented by macrophages (Endres & Grey 1980 a,b). In addition, the immune responsiveness of the animal can be altered by pharmacological manipulation prior to, during or after antigen administration.

I. Modulation by cyclophosphamide

Cyclophosphamide, an alkylating agent, has been used in a variety of experimental systems with variable effects.

When it is given shortly after immunisation, the predominant result is a dose related suppression of the immune response and affects mainly antibody synthesis (Mitsuoka, Baba & Morikawa 1976, Ramshaw, Bretscher & Parish 1976). If given before antigen, antibody responses are mainly unaffected and CMI may be enhanced by the suppressive action of cyclophosphamide on T-suppressor cells (Lagrange, Mackaness & Miller 1974, Asherson et al 1977, Gill & Liew 1978, Mitsuoka, Morikawa, Baba, & Harada 1979) Furthermore, cyclophosphamide pretreatment has been used to induce CMI responses locally in the gut associated lymphoid tissues (Mowat & Ferguson 1981) by concomitantly abrogating systemic tolerance which is dependent on T-supressor cells. Thus, cyclophosphamide is a useful tool for analysis of the immunoregulation of oral tolerance and will be used in the experiments described in this thesis.

II. Immunomodulation by immaturity

Although it has been shown that the neonatal period is exceptionally sensitive to tolerance induction by allogeneic cells (Billingham, Brent & Medawar 1953, Medawar 1961), by intravenous injection of human gamma globulin (Waters, Pilarski, Wegmann & Diener 1979, Chiller, Titus & Etlinger 1979), or by transfer of a tolerogenic form of human gamma globulin via colostrum (Halsey & Benjamin 1976) there is no report of induction of oral tolerance in neonatal mice except by Hanson (1981) where he used human gamma globulin. Indeed, it seems that the physiological route of dietary antigen exposure during the neonatal period has been neglected and recent reviews on the development of the immune system and the immunoregulatory capacity of the rodent foetus and newborn (Murgita & Wigzell 1981) do not mention any work dealing with the immune responses induced by feeding antigen during the neonatal period. Modulation of the immune response by antigen feeding during the neonatal period, however, seems to be of crucial importance in understanding the underlying mechanisms which tip the balance from tolerance to sensitisation and which could thus lead to food allergic diseases in humans.

E. AIM OF THE THESIS

This thesis will focus on experiments on the ways of induction and modulation of oral tolerance and examine whether abrogation of oral tolerance leads to local intestinal CMI responses. The experiments are conducted in inbred mice and ovalbumin was chosen as a dietary antigen for immunological (not related to self antigens) and practical

reasons (readily available ovalbumin-free mouse diets).

The specific objectives addressed in the thesis are:

1. To test and establish protocols and methods for assessment of systemic tolerance and induction of local mucosal cell mediated immunity.
2. To examine ways of abrogation of oral tolerance by immunomodulation with:
   (i) cyclophosphamide
   (ii) muramyl-dipeptide
   (iii) graft-versus-host reaction, and
   (iv) by perinatal ovalbumin feeding
3. To investigate the role of the gut in induction of oral tolerance by adoptive transfer of serum of fed donor mice.
4. To test whether abrogation of oral tolerance leads to local intestinal cell mediated immunity on antigen re-exposure in later life.

The long term aim of the work presented here is to establish an animal model for human food allergic diseases so that methods for their prevention and their reversal can be based on knowledge of the underlying immunological mechanisms.

Chapter 2

MATERIALS AND METHODS

A. ANIMALS

Mice of the following inbred strains were used throughout the experiments:

CBA/Ca ($H/2^{k/k}$) and BALB/c ($H/2^{d/d}$) originated from the Jackson Laboratories, USA, and have been bred since 1974 in the Animal Unit of the Western General Hospital, Edinburgh. (CBA x BALB/c) F_1 ($H/2^{k/d}$) and (C57BL/6 x DBA/2) F_1 ($H/2^{b/d}$) were bred on demand (CBA x BALB/c) F_1 or bred continuously (BDF_1). New Zealand white rabbits (NZW) were used to raise the anti-ovalbumin antisera.

The age of the animals was as indicated in the experimental section. Adult animals were mainly used at 6-8 weeks of age. They were kept on a 12/12 hour light/dark cycle and experimental procedures were carried out usually between 9.00 - 11.00 am.

B. DIET

Animals were fed a standard rodent diet (Spratts No.1, Spratts Patent Limited, Cambridge) and had ad libitum access to tap water.

In experiments in which animals were chronically challenged with ovalbumin, 2 mg ovalbumin/100 ml was dissolved in their drinking water equivalent to a daily intake of 0.1 mg/mouse, assuming a 5 ml daily fluid intake for an adult animal.

C. ANTIGENS

Ovalbumin, human serum albumin and bovine serum albumin, five times recrystalised, were obtained from Sigma Chemical Company, Poole, Dorset. Antigens were dissolved in sterile water or 0.15 M saline before injection or feeding.

D. ANAESTHESIA

Ether anaesthesia was used for procedures such as footpad immunisation, retro-orbital and axillary bleeding.

Abdominal surgery was carried out under barbital anaesthesia using SAGATAL 60 mg/ml (May and Baker Ltd) diluted 1:10 in sterile water. Mice were injected intraperitoneally with 0.01 ml/g body weight which provided adequate anaesthesia after about 15 minutes, lasting for up to two hours.

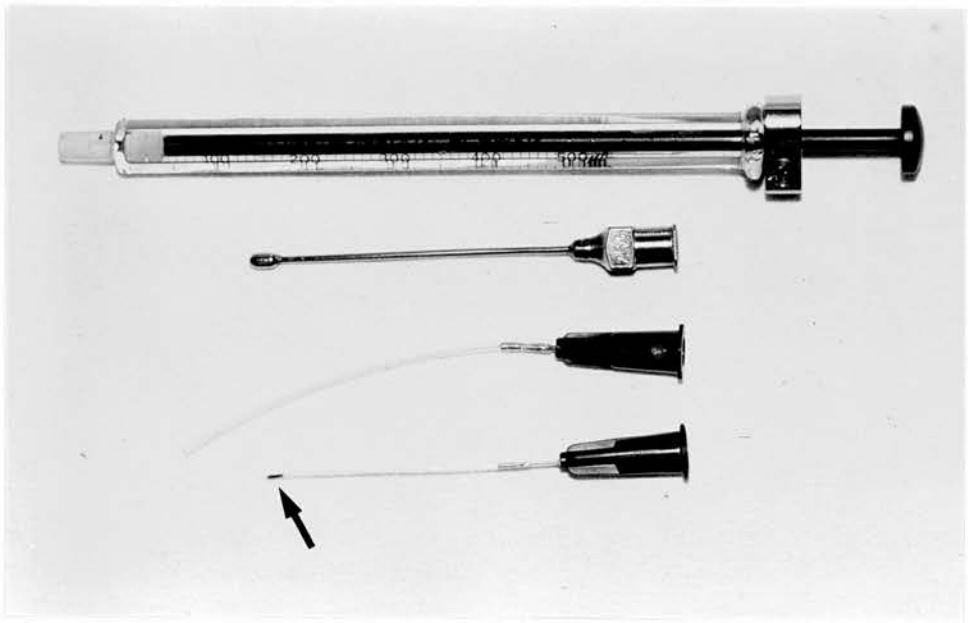
E. INTRAGASTRIC ADMINISTRATION OF ANTIGEN

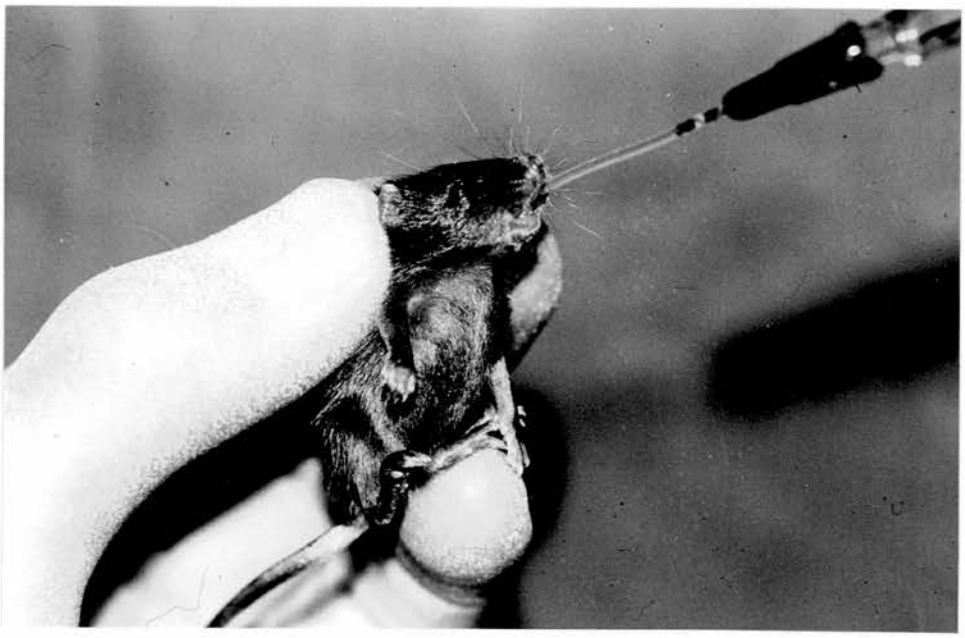
Experimental animals were not fasted. 100 mg ovalbumin was usually dissolved in 1 ml of 0.15 M saline thus providing the tolerising dose of 1 mg ovalbumin/g body weight in 0.01 ml/g body weight.

In experiments where dose dependency of tolerance induction was examined, the concentration of ovalbumin was increased and the maximal adminstered volume did not exceed 0.3 ml in adult or 0.05 ml in neonatal mice (age 1-14 days).

I. **Feeding technique and feeding equipment (Figures 2.1 and 2.1a)**

Mice aged three weeks and older were intubated with a 19 gauge stainless steel dosing needle with a spherical shaped blunted end. Experimental animals aged between two and three weeks were intubated with a premature infant feeding catheter (Argyle, Sherwood Medical Industries, 3.5 Charriere) attached to a 19 gauge stainless steel needle stock.





2.1 **Feeding techniques and equipment**

Top: A microsyringe (500 µl) and three feeding tubes used at different ages. The stainless steel tube with a blunted end was used after three weeks of age. Below, feeding tube used in early experiments (1-14 days), bottom: definitive feeding tube with a flexible, soft silicone rubber tip used from 1-14 days of age (arrow indicates the joining stainless steel needle stock). **Bottom:** Feeding technique and position demonstrated on a 14 day old BDF_1 mouse.

2.1a **Feeding techniques and equipment**

Test feed with Evan's blue of a one day old BDF_1 mouse. Note the clearly visible milk filled stomach on the right which now exhibits blue colouration (left) after feeding 0.05 ml Evan's blue solution. Perforation leads to a diffuse colouration of the abdomen.

Some of the neonatal mice up to 14 days were intubated with a 00 flex nylon intravenous tubing attached to a 26 gauge needle stock. To prevent mucosal abrasion and to minimise trauma, the end of the tubing was blunted by careful heating and checked for smoothness under the dissecting microscope.

A modified feeding tube with a flexible silicone rubber tip (No 602-105, Dow Corning, USA) was used in most of the neonatal feeding experiments. The smooth rubber tip was attached to polyvinyl tubing (Portex Limited) by connecting the two tubes with a circa 2 mm stainless steel stock of a 27 gauge needle which was inserted half into each tubing under the dissecting microscope. The soft tip was cut 1.5 mm above the joining piece.

All feeding techniques were checked before starting a series of experiments by feeding Evan's blue (Figure 2.1a). On dissection, the oesophageal and gastric mucosae were checked for injury and the pharynx, oesophagus and lungs were checked for reflux, injury, and <u>via falsa</u> intubation. No signs of injury, reflux or intratracheal intubation could be found after having established these experimental procedures. Animals which exhibited signs of regurgitation or bleeding were excluded from the experiments. The exclusion/mortality rate in mice fed at day one of life was less than 4%.

F. IMMUNISATION OF ANIMALS

In early studies of systemic immunity, mice were immunised with 2 mg ovalbumin suspended in complete Freund's adjuvant in a volume of 0.2 ml intraperitoneally. In all other experiments 100 µg antigen suspended in an equal amount of complete Freund's adjuvant was injected intradermally into one rear footpad (volume 0.05 ml).

NZW rabbits were immunised with 2 mg ovalbumin or human serum albumin emulsified in complete Freund's adjuvant intramuscularly at monthly intervals for three months and bled out two weeks after the last booster injection.

G. BLOOD COLLECTION

Up to 200 µl of blood were obtained routinely from the retro-orbital plexus under ether anaesthesia using heparinised hematocrit tubes (Propper Ltd, Long Island, New York). This route was chosen for survival experiments. Larger quantities of blood were obtained by either cardiac puncture or bleeding mice from the axillary vessels. Haematocrit tubes were sealed with CRISTASEAL (Hawskley, England) and allowed to clot in the upright position. They were spun in a haematocrit centrifuge (Hawskley, England) for five minutes, decomplemented for 30 minutes (water bath) at 56°C and stored at -20°C until assayed.

H. PASSIVE HAEMAGGLUTINATION ASSAY

Sera were tested for antibodies by passive haemagglutination. Sheep red blood cells were collected and stored in Alsever's solution (v/v) and washed three times in saline at 1500 x g before use. 100 µl packed sheep red blood cells were mixed with 0.7 ml saline and 100 µl protein solution (15 mg/ml ovalbumin or 1 mg/ml human serum albumin) in saline. 1 ml of 0.01% chromic chloride (Analar, BDH Limited) in saline at pH 5.0 was added dropwise under continuous agitation. The mixture was then allowed to stand for 10 minutes at room temperature (around 25°C),

the reaction was stopped by adding 10 ml of phosphate buffered saline pH 7.2 and the coated cells were then washed twice in phosphate buffered saline and resuspended at 1% before use.

Decomplemented sera were absorbed with 10% sheep red blood cells for about one hour before use. 25 µl serum was doubly diluted with 0.15 M saline in round bottom microtitre plates (TITERTEK, Flow Laboratories) and 25 µl of coated sheep red blood cells were added with a multi channel (eight) pipette (TITERTEK, Flow Laboratories) to each well.

After settling for 90 minutes at room temperature, the titres were taken as the last dilution to show complete agglutination. All sera were tested with or without the addition of 25 µl of 0.1 M 2-mercapto-ethanol (Sigma Chemical Co Ltd) to obtain information on both mercaptoethanol resistant and sensitive (presumably IgM) antibodies.

In some experiments, haemagglutination assays were performed in collaboration with Mrs H Drummond and Mrs M Gordon.

I. ASSESSMENT OF SYSTEMIC DELAYED TYPE HYPERSENSITIVITY

Mice were tested for delayed type hypersensitivity with microcalipers (POCOTEST-A, Carobronze Limited, London, United Kingdom) by measuring the specific footpad thickness increment 24 hours after an intradermal injection of 100 µg of antigen in 0.05 ml of saline into the plantar side of a non-immunised rear footpad. Control mice were either immunised with complete Freund's adjuvant and saline and tested with antigen, or were immunised with antigen in complete Freund's adjuvant and were tested with 0.05 ml of saline.

EX BIBL. UNIV. EDINBURGEN

J. SACRIFICE OF ANIMALS

Mice were killed by cervical dislocation.

I. Removal of tissues

Immediately after death, pieces of jejunum (5 mm) were removed 10 cm beyond the pylorus, avoiding Peyer's patches. They were placed on a card, cut open and immersed into the fixative, villus surface upwards.

II. Body weights

Body weights were obtained on living animals using an Oertling TD30 single pan balance.

III. Spleen weights

Spleens were dissected free of surrounding tissues after removal of the intestinal specimens, placed in an airtight container to prevent drying and were weighed on a torsion balance (White Electrical Instrument Company).

K. FIXATIVES AND HISTOLOGY

For conventional histology, jejunum and Peyer's patches were fixed in 10% buffered formalin, embedded in paraffin wax and sections were cut 5 microns thick. They were stained with haematoxyline and eosin. As preservation of intestinal mast cells or granulated intraepithelial lymphocytes is dependent on the fixative (Enerbäck 1966a) pieces of jejunum were removed as before, fixed in Carnoy's solution and stained

with astra-blue/safranine (BDH Limited) pH 0.3 (Bloom & Kelly 1960). Specimens were examined under a Leitz EB20 or Leitz Ortholux II microscope.

Histological processing was carried out by Mr Alexander Sutherland and Mr Frances Donnelly.

I. Intraepithelial lymphocyte counts

Intraepithelial lymphocyte counts are expressed as the number of intraepithelial lymphocytes/100 villus epithelial cells (Ferguson & Murray 1971). Sections were examined under x 1000 (oil immersion) magnification and only well cut sections with a single epithelial cell layer were counted.

Counts were done by enumerating epithelial and lymphoid cell nuclei lying unequivocally above the basement membrane and a total of 500 cells were counted in each specimen. In serially sectioned specimens care was taken to avoid recurring areas.

II. Counts of astra-blue positive granulated cells

Mucosal mast cells and cells with astra-blue positive granules within the epithelial layer were included in the total count. The cells were counted by using a square-grid eyepiece fitted to a Leitz EB20 microscope which was calibrated to give the number of cells per square millimetre of mucosa. The eyepiece was aligned with the lower edge lying on the upper surface of the muscularis mucosa close to the bottom of the crypts. The tissue was examined under x 400 and x 1000 magnification and 10 randomly chosen, well orientated sections were counted and the numbers expressed as cells/mm^2 mucosa. No correction was made for the area which was covered by the epithelium. Because of the distribution of these cells

in the lower one-third of the mucosa, there were no significant differences in counts obtained per square millimetre or per mucosal unit.

L. TISSUE PROCESSING FOR MICRODISSECTION (CLARKE 1970)

Mice were injected with 7.5 mg/kg colchicine (BDH Limited) intraperitoneally and sacrified at different intervals from 20-120 minutes after injection. Pieces of jejunum, 10 cm beyond the pylorus were removed, placed on cards, cut open and fixed in Clarke's fixative for maximum 24 hours. Thereafter they were transferred to 75% ethanol for storage before microdissection. To allow repeated examination in case of technical difficulties, only half of the tissues were stained in bulk by the modified Feulgen reaction. Pieces of gut were immersed in 50% ethanol for 10 minutes, followed by tap water for 10 minutes and 7 minutes hydrolysis in 0.1 N HCl at 60°C. The tissue was then rinsed three times with tap water and stained with Schiff reagent (Difco Limited) for 20-30 minutes at room temperature and then kept in tap water (maximum 48 hours) for microdissection.

The lamina muscularis mucosa was removed under the dissecting microscope (x32, Zeiss Stereomicroscope 4B) and a single villus or thin segments of mucosa containing a few villi and their crypts were then cut out of the non-traumatised mucosa by dissection with a cataract knife (Weiss Limited). The mucosal fragments were placed on a slide in a drop of 45% acetic acid, covered with a coverslip and examined under a microscope with a previously calibrated eyepiece micrometer.

In each specimen, the lengths of 10-15 complete villi and crypts were measured and the means taken for group comparisons. The tissue under the

coverslip was then gently squashed with a wooden stick and the number of arrested metaphases counted for at least 10-15 complete crypts.

I. **Crypt cell production rate**

The crypt cell production rate/hour was obtained by correlating the mean number of metaphases/crypt with the corresponding time interval after colchicine injection and submitting them to linear regression analysis. After having established linearity, the crypt cell production rate is calculated from the gradient of the line of best fit calculated by the method of least squares. Crypt cell production rates are given per hour with the correlation coefficient which indicates the relationship between the two variables; where there is complete correlation the value is 1.0; absence of correlation is indicated as 0.

M. PREPARATION OF SPLEEN CELL SUSPENSIONS

Spleens were removed immediately after sacrifice, dissected free of surrounding tissue and cut into little segments in cold RPMI 1640 medium (Flow Laboratories). The pieces were then passed through a stainless steel 60 gauge wire mesh with a plunger of a 5 ml plastic syringe (BD Limited). The resultant suspension was passed once gently through a 23 gauge needle to break up lumps. The cells were then washed three times at 400 g in cold RPMI 1640 until the supernatant was clear. Cell counts were done in a haemocytometer (improved Neubauer chamber) with white cell counting fluid. The cell pellet was made up to the required concentration in RPMI 1640 and injected into the recipients within 60 minutes after removal of the spleen. Cell viability as assessed by trypan blue exclusion was generally greater than 90% (range 82-96%).

N. INDUCTION OF GRAFT-VERSUS-HOST REACTION

Adult female animals of both F_1 hybrid generations (CBA; C57BL/6) were used for induction of GvHR. Adult animals received 6 x 10^7 CBA spleen cells in 0.2 ml of medium intraperitoneally.

Neonatal BDF_1 animals aged 1-2 days received 1 x 10^7 C57BL/6 spleen cells intraperitoneally in 0.05 ml RPMI 1640. Control animals received either medium alone or equivalent numbers of syngeneic F_1 hybrid spleen cells.

I. Assessment of graft-versus-host reaction

Simonsen's (1962) spleen weight assay was used. Mice were weighed shortly before sacrifice, the spleen removed, weighed and the relative spleen weight expressed as mg spleen/10g of body weight. The Spleen Index is calculated after the following equation:

$$\frac{\text{relative spleen weight in mice with GvHR}}{\text{relative spleen weight in control mice}} = \text{Spleen Index}$$

O. STATISTICAL EVALUATION AND PRESENTATION OF RESULTS

In general, results are expressed as means ($\bar{x}$) $\pm$ 1 standard deviation (SD) or $\pm$ 1 standard error (SEM) of the mean, as stated in the legends to the figures.

In interexperimental or animal strain comparisons, results are expressed as percentage change of the experimental group compared to the appropriate control group.

Students t-test was used to compare differences in most cases. In experiments including haemagglutination assays, comparison was done on

$\log_{10}$ transformed titres using the t-test and by non-parametric analysis (Wilcoxon's Rank Sum test). In practice, similar signifance levels were obtained. Results of the enzyme-linked immunosorbent assay (ELISA) are expressed as absorbance (A^{405}) units and were compared by Rank Sum analysis as non-parametric distributions could not be ruled out.

Crypt cell production rates calculated by linear regression were compared by analysis of co-variance to examine the differences between the slopes of the best fit curves. All calculations were done on a Casio fx 180P or on a programmable desk top calculator from Texas Instruments (TI59C) which was connected to a printer.

P. LIST OF SOLUTIONS AND BUFFERS

Phosphate buffered saline pH 7.2 (1000 ml)

anhydrous Na_2HPO_4	1.50 g
anhydrous KH_2PO_4	0.43 g
NaCl	7.20 g

0.03 M Carbonate buffer pH 9.6 (Northeast Biomedical Ltd) (1000 ml)

$Na_2\ CO_3$	1.59 g
$NaH\ CO_3$	2.93 g
NaN_3	0.20 g

diluted 1:1 with distilled water

10% Diethanolamine buffer pH 9.8 (1000 ml)

diethanolamine	100.0 ml
distilled water	840.0 ml
$MgCl_2 \times 6H_2O$	0.102 g
NaN_3	0.200 g

adjust to pH 9.8 with 1 M HCl (approximately 60 ml/litre of buffer)

Washing solution for ELISA (1000 ml)

0.15 M NaCl	1000.0 ml
Tween 20	0.5 ml

Serum diluent for ELISA (1000 ml)

0.15 M NaCl	1000.0 ml
Tween 20	0.5 ml
NaN_3	0.2 g

Alsever's solution

dextrose	20.5 g
NaCl	4.2 g
sodium citrate	8.0 g

dissolve in 1000.0 ml of water, sterilize by membrane filtration (0.22 μm) and store at room temperature.

RPMI 1640 medium (500 ml)

tissue culture medium without glutamin. Flow Laboratories, Order No. 12-602-54.

Fixatives:

10% buffered formalin (1000 ml)

formalin (40% formaldehyde)	100.0 ml
distilled water	900.0 ml
$NaH_2PO_4 \times 2H_2O$	4.0 g
Na_2HPO_4	6.5 g

Clarke's fixative (1000 ml)

ethanol (96%)	750.0 ml
glacial acetic acid	250.0 ml

Carnoy's fixative (1000 ml)

ethanol (96%)	600.0 ml
chloroform	300.0 ml
glacial acetic acid	100.0 ml

(store in a dark, airtight glass container)

Chapter 3

NEW TECHNICAL DEVELOPMENTS

A. ENZYME LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF ANTI-OVALBUMIN-ANTIBODIES IN MOUSE SERUM

Measurements of serum antibodies by passive haemagglutination with sheep red blood cells, although a relatively simple and specific immunological method, has several disadvantages.

The test is dependent upon the availability of fresh sheep red blood cells, requires extensive washing and time-consuming coating procedures and the coating process is dependent on the age of the sheep red blood cells. Test sera have to be absorbed to remove heterophilic antibodies and serially diluted before testing. A further theoretical disadvantage is that IgM antibodies agglutinate antigen coated sheep red blood cells better than IgG antibodies which have a different antigen coating optimum. Furthermore, it seemed that the reproducibility and sensitivity in the low titre range was not as good as in medium and high titre (above 1:1280) sera. Thus, a less time consuming, specificity and sensitivity improved solid phase enzyme linked immunosorbent assay (ELISA) was developed. Following the general procedures as outlined in Voller, Bidwell & Bartlett (1979), chequerboard titrations and incubation studies were performed to determine optimal concentrations of antigen, antiserum, enzyme conjugates and substrate.

After setting up the ELISA assay, this newly developed test was then validated against sera which had already been tested by passive haemagglutination.

I. Setting up the anti-ovalbumin-antibody ELISA

Preliminary experiments showed that a macro-ELISA system was

workable but its reproducibility was unsatisfactory. Thus a micro-ELISA system using microtitre plates (LINBRO, Flow Laboratories) was set up and the following conditions were tested by chequerboard analysis:

Coating conditions:	4°C for 16 hours, 37°C for 2 hours
Coating concentrations:	0.1, 1.0, 5.0, 10.0 mg ovalbumin/ml in 0.03 M carbonate buffer, pH 9.6
Serum dilutions:	1/50 in doubling dilution steps to 1/6400
Serum diluent:	0.05% Tween 20 (BDH Limited) in 0.15 M NaCl 0.02% sodium azide
Serum incubation:	For 5 hours at room temperature
Conjugate dilutions:	1/500 - 1/2000
Conjugate incubation:	For 16 hours at room temperature
Substrate concentration:	1 mg/ml p-nitrophenylphosphate in 10% diethanolamine buffer

Each incubation step was followed a washing step which consisted of three washings with 0.05% Tween 20 in 0.15 M NaCl. The definitive anti-ovalbumin-antibody ELISA which was used throughout the study was as follows:

Working volume:	100 µl
Coating time:	2 hours at 37°C
Antigen concentration:	1 mg ovalbumin/ml
Serum incubation:	5 hours at room temperature
Serum dilution	1/100
Conjugate incubation:	16 hours (overnight) at room temperature
Conjugate dilution:	1/1000 (antiserum from Northwest Biomedical Limited) 1/2000 (antiserum from Miles Limited)

Each incubation step was followed by a washing step as indicated above.

Substrate incubation: Under the above conditions the positive control hyperimmune serum reached 1.0 absorbance after 30-45 minutes at room temperature. Absorbance was measured at 405 nm against a reference wavelength of 630 nm with an automatic microelisa reader (Dynatech Limited)

1. Variations of antigen binding to the solid phase

It has been noted that there is an apparent variation in the degree of antigen absorption to the surface of the wells in different sections of microtitre plates.

Absorbance was tested in empty plates and in plates in which all wells had been treated uniformly so that each well received the same dilution of antigen, serum, conjugate and substrate. In repeated experiments, less than 4% of the A^{405} readings within one plate were outwith one standard error of the mean absorbance reading of the whole plate, indicating a satisfactory uniform binding.

2. Assay controls

Each plate contained wells which received only serum diluent during each step in the assay. These wells received substrate and were used as blanks to zero the ELISA reader.

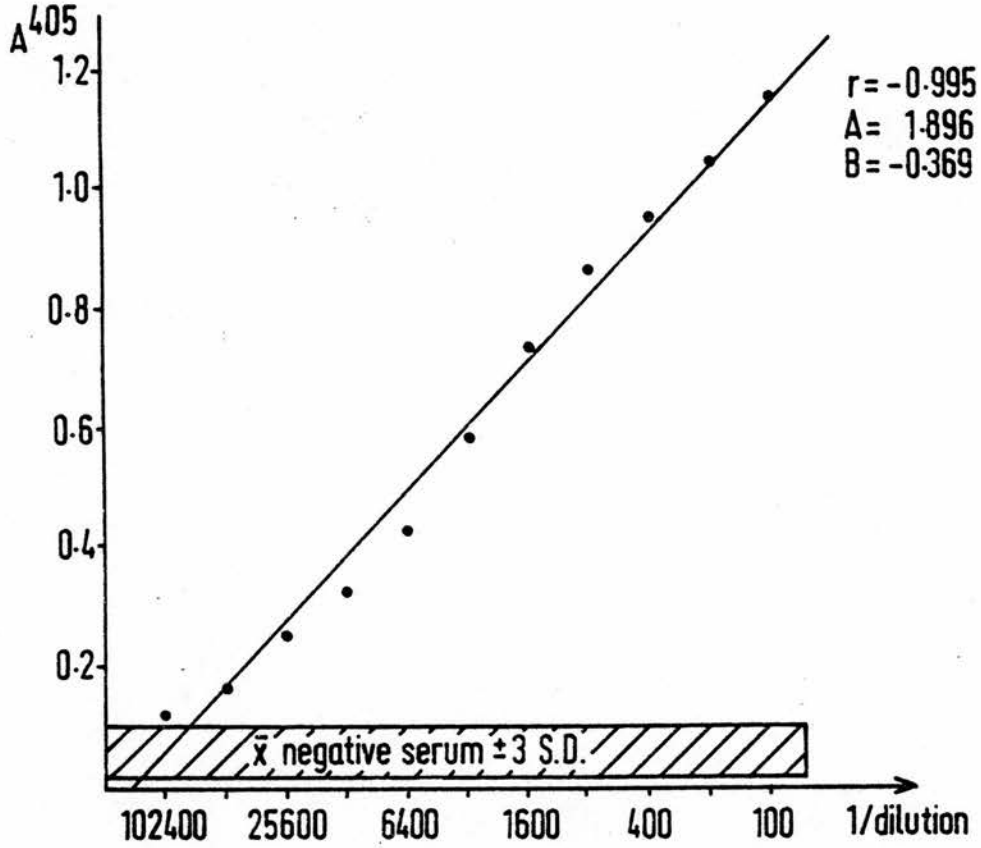
Conjugate controls (ie. wells coated with antigen, conjugate and

substrate, but without serum) were included to determine the non-specific binding of the conjugated antiserum to the antigen coated wells. In addition, each plate was run with appropriate dilutions of positive and negative reference sera. Each treatment condition was assayed in triplicate.

3. Testing for linearity of serial anti-serum dilutions

To investigate whether serum antibody concentrations or the antimouse antibody enzyme-conjugate were the rate-limiting factors in this test system, positive and negative reference sera were serially diluted from 1/100 - 1/102400 and assayed. Figure 3.1 shows an excellent correlation between reciprocal serum dilutions plotted against absorbance values ($r.995$, $p<0.001$) indicating that the concentration of antibody enzyme-conjugate was not the rate-limiting factor in the assay and that the linearity of this assay system covered almost three $\log_{10}$ units. Diluting the negative serum beyond 1/100 did not change the A^{405} readings significantly. In order to investigate whether the experimental group characteristics remained stable throughout different dilutions, titration studies with positive and negative reference sera were performed. Sera from a group of tolerant mice which had been fed ovalbumin before immunisation, and of a saline fed, non-tolerant, group were included.

The results demonstrated that there is no crossover and the positive/negative ratio (ovalbumin fed vs. saline fed animals) remains stable, indicating maintenance of group characteristics over a wide range of anti-ovalbumin antibody concentrations.



3.1 **Test for correlation between serum antibody concentrations and absorbance at 405 nm (ELISA)**

Serum containing anti-ovalbumin antibodies and normal mouse serum were serially diluted from 1/100 to 1/102400 and the absorbance recorded at 405 nm. Note the good correlation of the slightly S-shaped curve over a wide range of serum dilutions. Mean absorbance readings of the normal mouse serum (± 3SD) are indicated by the hatched area.

II. Comparison of ELISA with haemagglutination assay

Before changing to a new test system a comparison between groups tested with ELISA and the haemagglutination assay was carried out. 65 sera were tested by haemagglutination and by ELISA tests and the corresponding means of absorbance values and haemagglutination titres are plotted in Figure 3.2 and show a good correlation ($r = 0.930$, $p<0.001$).

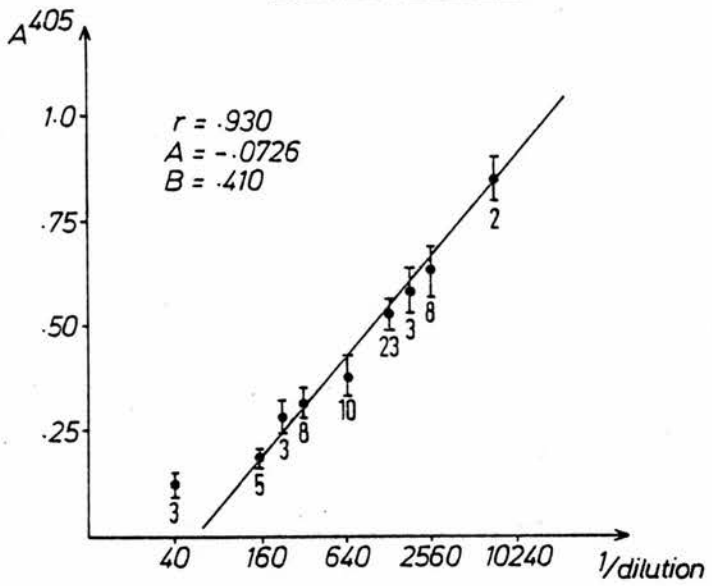
B. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF OVALBUMIN IN MOUSE SERUM

The need for a sensitive, reliable assay system became obvious when published reports (Warshaw, Walker & Isselbacher 1974, Vaz et al 1977, Swarbrick 1979, Mowat 1981) not only demonstrated highly variable amounts of ovalbumin after feeding, but that the amounts detected were also as low as 2 ng ovalbumin/ml serum one hour after feeding (Swarbrick 1979).

A passive haemagglutination inhibition test commonly used for detection of immunoreactive antigen was not likely to yield this sensitivity as was shown by Mowat (1981) working in the same laboratory. The detection limit was around 1-3 µg ovalbumin/ml serum.

To achieve the above stated objectives, two different approaches were pursued. One was based on the principle of a competitive (inhibitory) ELISA and a further on a direct ELISA (Engvall & Pesce 1978, Voller et al 1979) using protein-A purified (Hjelm, Hjelm & Söquist 1972) specific IgG-anti-ovalbumin antibody bound to the solid phase.

Correlation of A^{405} readings with passive haemagglutination titres ($\bar{x} \pm$ SEM) n=65



3.2 **Comparison of absorbance readings at 405 nm (ELISA) with passive haemmagglutination titres**

65 sera were tested by the two assay systems and the reciprocal haemmagglutination titres were plotted against absorbance (405 nm) readings ($\bar{x} \pm$ SEM). The figures below the bars indicate the numbers of sera tested in each group.

I. Direct ELISA ('Sandwich') for measurement of circulating ovalbumin after feeding

The inhibitory ELISA system did not reach the desired sensitivity (<20 ng ovalbumin/ml serum) and a different approach was used. To achieve these objectives, a direct (sandwich type) ELISA was set up in collaboration with Dr Don Hanson using specifically purified anti-ovalbumin antibodies of the G-class bound to the solid phase.

II. Isolation of IgG antibodies by protein-A sepharose CL4-B

Rabbit anti-ovalbumin antiserum (Miles Yeda Laboratory) was purified for G-class antibodies by protein-A sepharose (Pharmacia Fine Chemicals AB, Uppsala) which is the method of choice for isolation of IgG-type antibodies (Hjelm et al 1972). For the isolation procedure of G-class antibodies, the manufacturers instructions (Pharmacia Fine Chemicals AB, Uppsala) were followed and G-class antibodies were eluted with 0.3 M acetic acid.

III. Purification of specific IgG anti-ovalbumin antibodies by affinity chromatography

Ovalbumin specific antibodies are only a minor constituent of the total IgG antibody fraction even in hyperimmune sera and thus affinity chromatography with ovalbumin bound to sepharose was used to yield a preparation of IgG anti-ovalbumin antibodies.

Cyanogen bromide (CNBr) activated sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala) was used to couple ovalbumin covalently to sepharose following the manufacturers instructions ('Affinity chromatrography', Pharmacia Fine Chemicals AB, Uppsala 1979). In short:

1 g of gel (3.5 ml final gel volume) is swollen in posphate buffered saline pH 7.4 and washed for 15 minutes on a glass filter with 0.003 M HCl solution. 10 mg ovalbumin are dissolved in 0.1 M $NaHCO_3$ containing 5 ml of 0.5 M NaCl and then mixed with the gel by end over end rotation at room temperature avoiding magnetic stirrers.

Unbound material was washed away with the coupling buffer and remaining active groups were blocked with 1.0 M ethanolamine for 1-2 hours. Non-covalently absorbed ovalbumin was removed by three wash cycles with the coating buffer. Ovalbumin specific IgG class antibodies were eluted with 0.3 M sodium isothiocyanate (NaSCN). The buffer was exchanged by sephadex G-25 (Pharmacia Fine Chemicals AB, Uppsala) chromatography and elution with 0.15 M NaCl; 0.02% sodium azide was added to prevent bacterial growth. The concentration of IgG class ovalbumin specific antibody which was used in the ELISA was 10 µg/ml.

IV. Setting up the ELISA system

The following conditions, which were chosen according to my own experience and published guidelines (Voller et al 1979), were analysed by chequerboard analysis.

Solid phase:	microtitre plates, polystyrene (LINBRO, Flow Laboratories) acrylic plates (FALCON, Becton Dickinson)
Working volumes:	100, 125, 150 µl
Coating concentration:	1, 10 mg/ml IgG anti-ovalbumin-antibody solution in 0.03 M carbonate buffer pH 9.6
Coating conditions:	16 hours at 4°C, 2 hours at 37°C

Ovalbumin serum standards:	range: 0-10^5 ng ovalbumin in normal mouse serum
Incubation time:	16 hours at room temperature
Ovalbumin serum standard dilutions:	1/2.5, 1/5, 1/10/ 1/100 with serum diluent
Test serum dilution:	according to standard dilutions
Alkaline phosphatase conjugate dilutions:	IgG anti-ovalbumin specific antibodies coupled with alkaline phosphatase by the glutaraldehyde method (performed by Northeast Biomedical) 1/500, 1/1000, 1/2000, 1/4000

All plates were washed three times with saline and 0.05% Tween 20 between each incubation step and p-nitrophenylphosphate was used as substrate (1 mg/ml in 10% diethanolamine buffer). For the final test system, used in this thesis, the following conditions were used:

Working volume:	150 µl
Coating concentration:	10 µg/ml IgG anti-ovalbumin-antibody in 0.03 M carbonate buffer pH 9.6
Coating condition:	2 hours at 37°C
Ovalbumin serum standard dilutions:	1/10
Test serum dilution:	1/10
Alkaline phosphatase Conjugate dilution:	specifically purified IgG anti-ovalbumin coupled to alkaline phosphatase was used at 1/500 dilution
Conjugate incubation:	16 hours at room temperature

1. Test sensitivity

A typical standard curve is shown in Figure 3.3 and the sensitivity was increased more than ten-fold compared to the inhibitory ELISA. The detection limit was defined by taking two standard deviations above the mean absorbance reading of non-ovalbumin containing normal mouse serum as the lower limit of sensitivity. Thus, taking into account the day to day variation of the absorbance readings of normal mouse serum, the threshold of detection varied from 0.3 to 3 ng ovalbumin/ml serum.

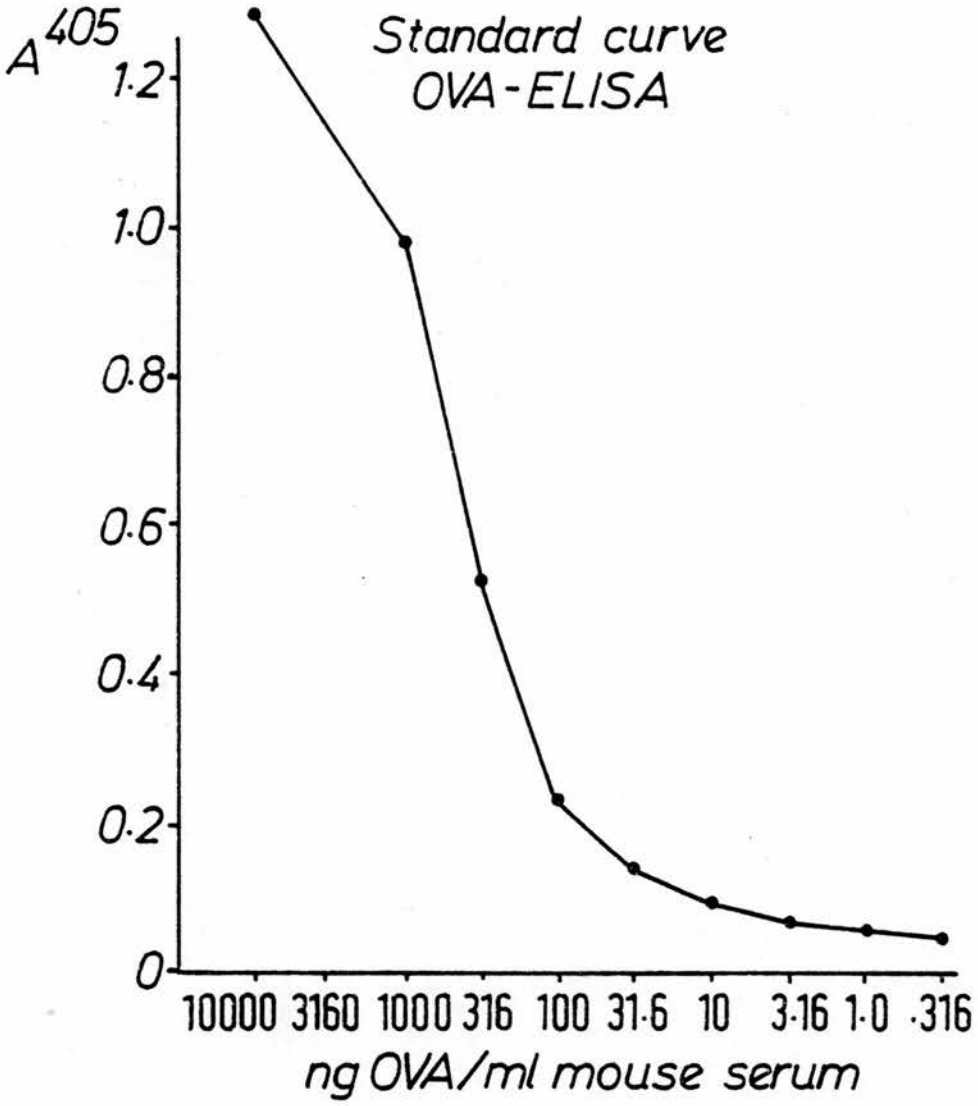
The coefficient of variation within the test was 4.6% (n = 8) and between tests 9.2%.

C. COMMENT AND DISCUSSION ON NEW TECHNICAL DEVELOPMENTS

The haemagglutination test using antigen coated sheep red blood cells is a standard and well defined immunological test system. However, the variation from batch to batch of sheep red blood cells, the time which is needed to perform absorption, the preparation of the microtitre plates and performing the dilutions and the lower sensitivity at lower antibody concentrations, make this test unsuitable for testing large experimental groups under identical conditions. Thus, ELISA assays for the detection of ovalbumin and of ovalbumin specific antibody were developed, extensively tested and shown to give a better distinction between treatment groups.

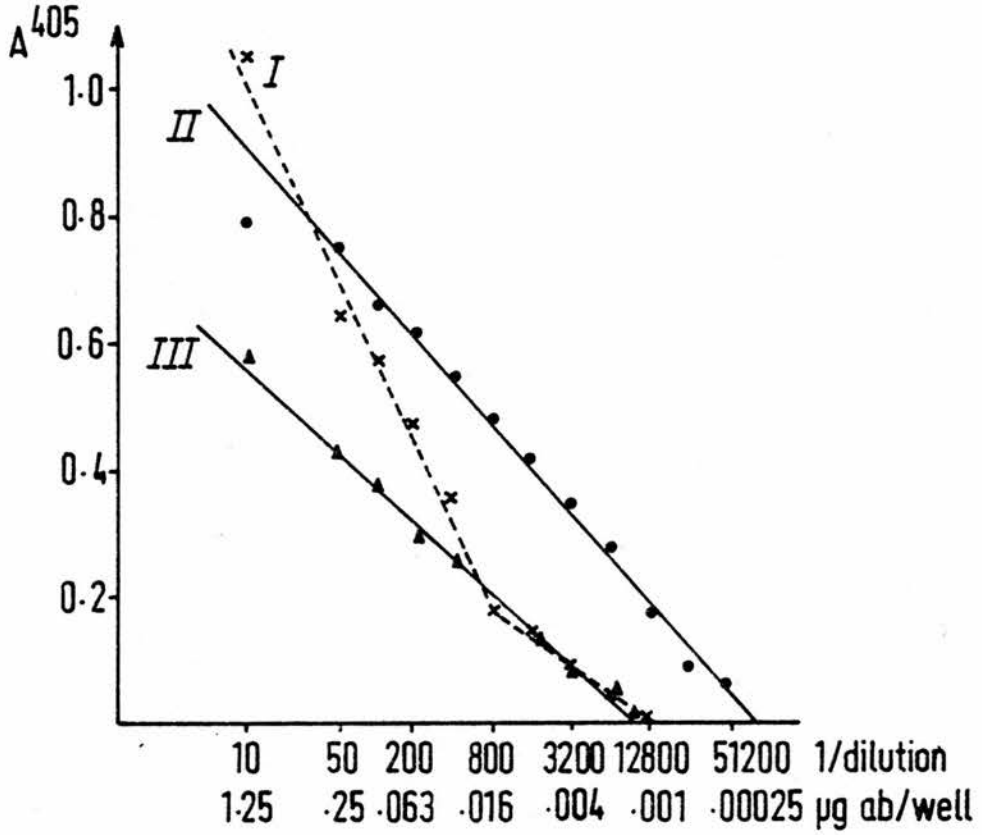
It is common practice to present the results of the ELISA test as A^{405} readings or as the ratio of positive/negative group readings (Voller et al 1979). However, this practice may be criticised and the optimal presentation of ELISA results would be by giving test results in

nanogrammes of specific antibody/ml serum. In a pilot experiment, I tested and compared serial dilutions of known amounts of specficially purified anti-ovalbumin antibody with unknown amounts of ovalbumin antibody of a mouse hyperimmune serum (Figure 3.4). It can be seen that the absorbance readings of the mouse hyperimmune serum were comparable to those obtained when purified IgG class anti-ovalbumin antibody was added to normal mouse serum. In this thesis all ELISA results will be presented as individual absorbance readings.



3.3 **Typical ovalbumin-ELISA standard curve**

Amounts of ovalbumin (in nanograms) added to normal mouse serum (logarithmic scale) are plotted against absorbance (405 nm) readings.



3.4 **Comparison of an anti-ovalbumin hyperimmune serum with known amounts of specifically purified anti-ovalbumin antibodies of the IgG-class**

Reciprocal serum dilutions and concentrations of specifically purified anti-ovalbumin antibody/well are plotted against A^{405} readings.

I : Purified anti-ovalbumin antibody diluted with serum diluent

II : Hyperimmune serum diluted with serum diluent

III: Specifically purified antibody (as in I) diluted 1/10 with normal mouse serum before further dilution with serum diluent.

Chapter 4

ORAL TOLERANCE TO OVALBUMIN AND ITS MODULATION

My first experiments were performed to assure that standard oral tolerance to fed antigen could be produced in the animal strains available for the experiments (BALB/c, BDF_1,CBA) and that this phenomenon was consistent, the induction reproducible and the systemic tolerance specific for ovalbumin.

The intitial protocol (Swarbrick 1979) required a 14 day period between feeding and immunisation and separate sites of immunisation for assessment of antibody responses and CMI responses. It is shown in Figure 4.1 and typical results in Figure 4.2. This protocol was modified in order to reduce the time scale and to reduce the numbers of animals required while retaining the capacity to measure the suppressed systemic immune response.

The final experimental protocol used for the investigation of oral tolerance was changed as follows:

1. Reduction of the time interval between feeding and immunisation to seven days;
2. By changing the route of immunisation to footpad immunisation, the same animals could be assessed for antibodies and CMI responses, thus reducing the numbers of animals and the variability of the test system.

The modulation treatments were broadly concerned with increasing T-help or reducing T-suppression and activating macrophages. The conditions are shown together with the general protocol in Figure 4.3. By applying antigen via an aboral route (rectal enema), Peyer's patches, a privileged site of antigen presentation (Owen 1977), and intraluminal digestion have been avoided. Finally, the modulating effects of a GvHR were investigated in a separate set of experiments.

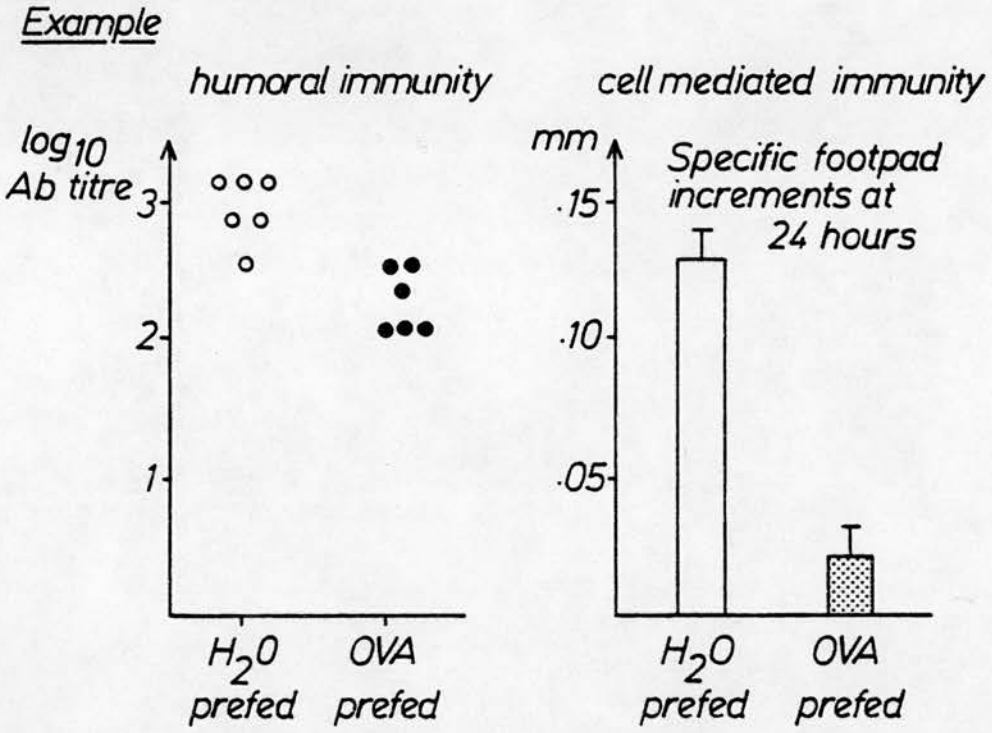
EXPERIMENTAL PROTOCOL

Group	Feed Day 1	Immunisation Day 14
Control	H_2O	a) 2mg OVA/CFA i.p. b) 100 µg OVA/CFA i.d.
Tolerant	25mg OVA	a) 2 mg OVA/CFA i.p. b) 100 µg OVA/CFA i.d.

mice immunised after a): bled weekly for antibodies (days 21, 28, 35)
mice immunised after b): tested for systemic DTH at day 35

4.1 **Initial experimental protocol**

The initial experimental protocol required a 14 day interval between feeding and immunisation. Groups of mice were immunised after a) for antibodies and after b) for tests of cell mediated immunity.



4.2 **Example: Humoral and cell mediated immunity in ovalbumin-fed and control animals**

Haemagglutinating antibody titres are given on a logarithmic (log_{10}) scale; footpad increments in millimetres (3 weeks after immunisation).

A. INDUCTION OF ORAL TOLERANCE TO OVALBUMIN IN DIFFERENT STRAINS OF MICE

I. Suppression of systemic immune responses

To demonstrate that oral tolerance to ovalbumin is not confined to a certain strain of mice, BALB/c, BDF_1 and CBA mice were treated according to the general experimental protocol (Figure 4.3) and systemic immune responses assessed three weeks after immunisation. The results for repeated experiments in adult female mice are listed in Table 4.4. They show that the suppression of antibody and CMI responses does not vary significantly between the different strains of mice, with 70-95% suppression of antibody responses and 78-96% suppression of CMI responses.

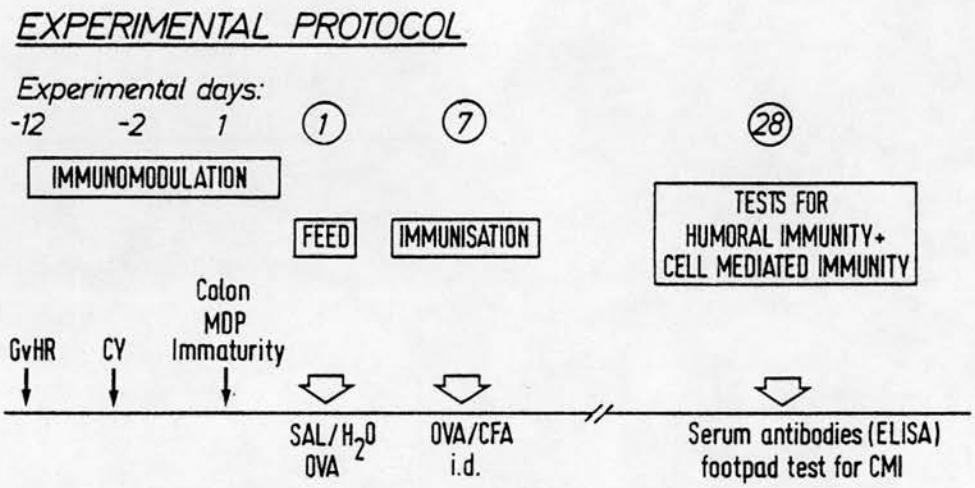
II. Specificity of systemic tolerance induced by ovalbumin

To confirm that the state of tolerance induced by feeding ovalbumin was immunologically specific to the fed protein, mice were immunised with 2 mg of human serum albumin in complete Freund's adjuvant two weeks after feeding 25 mg ovalbumin and tested one and three weeks later for antibodies and three weeks later for CMI responses.

Figure 4.5 shows that feeding of ovalbumin did not alter the immune responsiveness against human serum albumin and that ovalbumin fed mice exhibited similar antibody and footpad responses to their water fed controls.

III. Persistence of tolerance to ovalbumin

Persistence of tolerance to self antigens seems to be dependent on



indicate the essential steps which form the basis for all subsequent experimental protocols

IMMUNOMODULATION

GvHR	*Graft versus host reaction*
CY	*Cyclophosphamide i.p.*
MDP	*N-acetyl-muramyl-Dipeptide*
Immaturity	*OVA feeds during perinatal period*
Colon	*Colonic OVA adminstration*

6-8 ♀ mice/experimental group

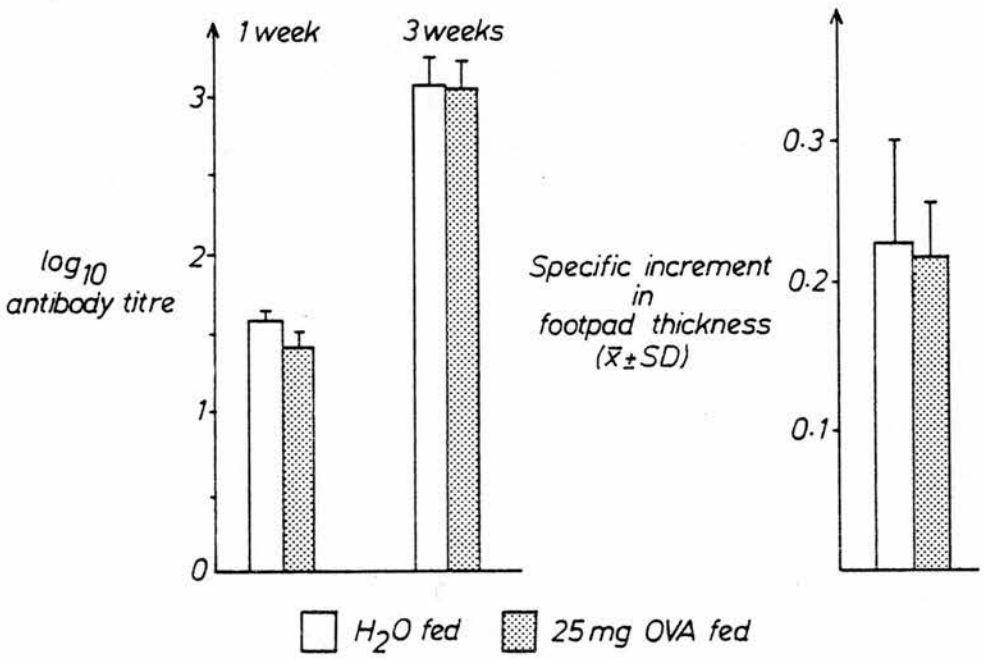
4.3 General experimental protocol

This graph shows the experimental protocol for the investigation of oral tolerance and for the effects of immunomodulation. The day of feeding is assigned "day 1" and all other times indicated are related to this day.

Table 4.4

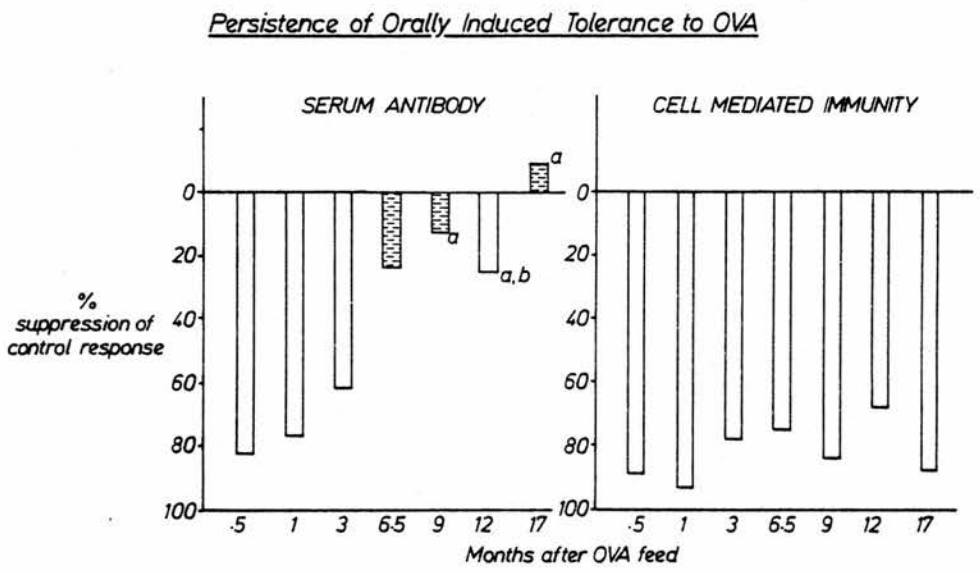
Induction of systemic hyporesponsiveness (oral tolerance) in different strains of mice

Feed	Humoral immunity ($\log_{10}$ haemagglutination titres) ($\bar{x} \pm$ SD) saline	ovalbumin	% suppression	Cell mediated immunity Specific footpad swelling(mm) ($\bar{x} \pm$ SEM) saline	ovalbumin	% suppression
CBA	3.08 ± 0.14	2.12 ± 0.22	78	0.12 ± 0.01	0.022 ± 0.01	82
	3.21 ± 0.21	2.39 ± 0.31	85	0.16 ± 0.01	0.011 ± 0.01	93
	3.11 ± 0.18	2.56 ± 0.28	70	0.11 ± 0.01	0.013 ± 0.01	78
BALB/c	2.96 ± 0.18	2.16 ± 0.24	84	0.18 ± 0.01	0.018 ± 0.02	90
	3.04 ± 0.20	2.54 ± 0.16	80	0.14 ± 0.02	0.006 ± 0.01	96
	3.42 ± 0.32	2.32 ± 0.31	92	0.16 ± 0.03	0.014 ± 0.01	81
	3.14 ± 0.26	2.29 ± 0.34	86	0.12 ± 0.01	0.017 ± 0.02	86
BDF_1	3.11 ± 0.22	1.81 ± 0.31	95	0.11 ± 0.01	0.023 ± 0.02	79
	3.18 ± 0.18	2.26 ± 0.18	88	0.14 ± 0.02	0.008 ± 0.01	94
	3.06 ± 0.20	2.38 ± 0.16	79	0.15 ± 0.01	0.020 ± 0.01	87



4.5 Specificity of systemic tolerance induced by feeding ovalbumin

Anti-human serum albumin antibody responses measured one and three weeks after immunisation with human serum albumin in Freund's complete adjuvant in mice fed 25 mg ovalbumin before immunisation ($\bar{x}$ + SEM). Systemic delayed hypersensitivity responses three weeks after immunisation with human serum albumin with Freund's complete adjuvant in mice fed ovalbumin as above. Bars represent mean (+ SD) specific footpad increments at 24 hours after challenge with 100 μg human serum albumin (i.d.). Ovalbumin feeding did not suppress immune responses against human serum albumin.



4.6 **Persistence of orally induced tolerance to ovalbumin**

Serum antibody and CMI responses in BDF_1 mice which were fed ovalbumin or saline at six weeks of age and immunised at various times thereafter. Immune responses were measured three weeks after immunisation. Stippled bars indicate the absence of significant suppression.

a) measured with ELISA

b) significant suppression ($p<0.05$) when compared to controls.

Table 4.7

Persistence of orally induced tolerance to ovalbumin

		Ab responses ($\bar{x} \pm SD$)			CMI responses ($\bar{x} \pm SEM$)		
	Feed		Suppression	p<		Suppression	P<
2 weeks	SAL	3.27 ± 0.21		0.01	0.18 ± 0.01		0.001
	OVA	2.61 ± 0.19	78%		0.02 ± 0.01	89%	
4 weeks	SAL	3.14 ± 0.25			0.15 ± 0.02		0.01
	OVA	2.51 ± 0.20	77%	0.01	0.011 ± 0.008	93%	
3 months	SAL	3.42 ± 0.32			0.21 ± 0.02		0.001
	OVA	3.00 ± 0.25	62%	0.05	0.046 ± 0.001	78%	
6.5 months	SAL	0.512 ± 0.047			0.11 ± 0.013		
	OVA	0.442 ± 0.068	14%	NS	0.028 ± 0.008	75%	0.005
9 months	SAL	0.683 ± 0.05		NS	0.13 ± 0.017		
	OVA	0.598 ± 0.048	12%		0.02 ± 0.019	84%	0.001
12 months	SAL	0.925 ± 0.51			0.11 ± 0.015		
	OVA	0.690 ± 0.23	25%	0.05	0.018 ± 0.007	68%	0.005
17 months	SAL	1.1 ± 0.08			0.12 ± 0.016		
	OVA	1.2 ± 0.06	+9%	NS	0.02 ± 0.012	83%	0.005

Experimental data which formed the basis for Figure 4.6. "6.5 months" group was also tested by haemagglutination assay and the suppression was not significant (23%, pNS).

persistence of the antigen (Dresser & Mitchison 1968, Nossal & Pike 1980). Tolerance after injection of human gamma globulin affects the T and B-cell systems differently (Chiller & Weigle 1971, Weigle 1977), lasting 2-3 months for the B-cell system and approximately 5-6 months for the T-cell compartment.

Persistence of oral tolerance to protein antigen has not been systematically studied and in published reports, tolerance for humoral responses has persisted for around 60 days (Vaz et al 1977, Ngan & Kind 1978, Challacombe & Tomasi 1980) and for six months in delayed type hypersensitivity responses (Kagnoff 1978a).

1. Experimental protocol

Groups of age matched female mice were fed ovalbumin or saline on the same day and immunised with ovalbumin in complete Freund's adjuvant, .5,1,3,6,9,12,17 months thereafter.

The results are depicted in Figure 4.6 and Table 4.7. They demonstrate clearly that the humoral immune response is significantly suppressed over three but less than 6.5 months after feeding, whereas the CMI responses are still signficantly suppressed 17 months after the initial feed.

IV. **Comment**

Since all control animals were age matched, the obtained results are not likely to have been affected by the reduction of the immune responsiveness of ageing animals and are due to the intitial treatment. The results show that T and B-cell compartments are differently affected as has been demonstrated after intraveneous injection of human

gamma globulin (Weigle 1977, Waters et al 1980). My observations also show that systemic tolerance induced by antigen feeding is a long lasting event in BDF_1 mice.

B. MODULATION OF ORAL TOLERANCE TO OVALBUMIN BY CYCLOPHOSPHAMIDE AND A LOW DOSE TOLERISING SCHEDULE

Feeding of antigen to rodents is more likely to induce systemic tolerance than active immunisation. One way to investigate the underlying mechanisms of oral tolerance is to modulate the induction phase by specific immunomodulating treatments and then to assess the effects of this treatment on oral tolerance.

Cyclophosphamide has been shown to increase CMI responses (Turk, Parker, Poulter 1972, Lagrange et al 1974) and in the following years it has been demonstrated that when given before antigen, it prevents the generation of T-suppressor cells from short lived precursors but does not affect the mature effector T_{DTH} or cytotoxic T-lymphocytes (Röllinghoff et al 1977, L'age-Stehr & Diamantstein 1978). T-suppressor cells have been confirmed to be more susceptible to active metabolites of cyclophosphamide than B or T-helper cells (Kaufmann, Hahn & Diamantstein 1980). Cyclophosphamide has also been shown by Mowat and Ferguson (1981) to be capable to induce a state of CMI to a dietary protein. Since suppressor T-cells have been implicated in induction of oral tolerance by feeding ovalbumin (Richman et al 1978, Ngan & Kind 1978, Miller & Hanson 1979), I wanted to investigate whether cyclophosphamide pretreatment would abrogate systemic tolerance observed after feeding. This information would be important for the evaluation of the immunoregulatory role of T-suppressor cells.

Systemic immunity and local CMI may differ in their susceptibility to intravenous tolerance induction (Silver & Benacerraf 1974) and they may therefore be controlled by different regulatory mechanisms which could be activated in a dose dependent manner.

The results of the long term experiments described in the previous chapter point in the same direction. Thus the following set of experiments were designed to give information on the regulation of humoral and cell mediated immunity and to clarify the potential role of the T-suppressor cell. Cyclophosphamide was used in a dose of 100 mg/kg and was administered intraperitoneally two days prior to feeding of antigen.

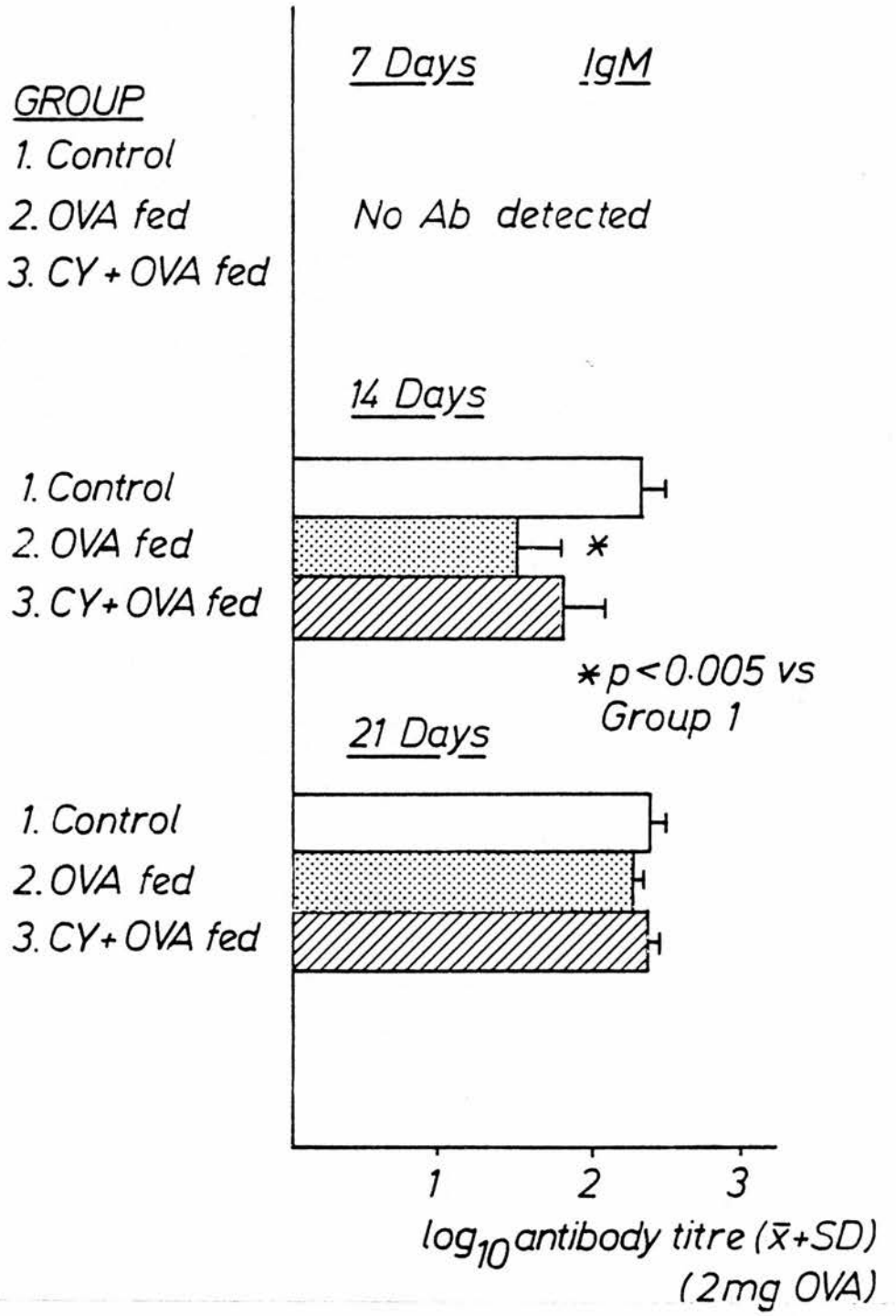
I. Experimental protocol and results

The experiments were performed in BALB/c mice according to the experimental protocol shown in Figure 4.3. Control groups received water intraperitoneally and orally and further control groups received water intraperitoneally and were fed 2 or 25 mg ovalbumin. A third group received cyclophosphamide intraperitoneally and was fed 2 or 25 mg ovalbumin two days later. After 14 days all groups were immuninised with ovalbumin in complete Freund's adjuvant.

1. Humoral antibody responses

Figure 4.8 shows the antibody responses 7,14 and 21 days after primary immunisation for animals which were fed 2 mg ovalbumin before immunisation. No antibodies were detected at seven days and at 14 days the ovalbumin fed group showed a suppression of 60% of their antibody responses ($p<0.05$). This suppression was partially reversed after cyclophosphamide treatment. The antibody titres were 2-mercaptoethanol sensitive and thus presumably of the IgM class.

Immunomodulation by CY pretreatment



4.8 **Immunomodulation of oral tolerance by pretreatment with cyclophosphamide**

Humoral antibody responses in mice which were fed 2 mg ovalbumin two days after injection of 100 mg/kg cyclophosphamide (see 4.2 and 4.3). There was no suppression of the systemic antibody responses three weeks after immunisation.
Note a transient suppression of 2-mercaptoethanol sensitive antibody levels (IgM) after 14 days.

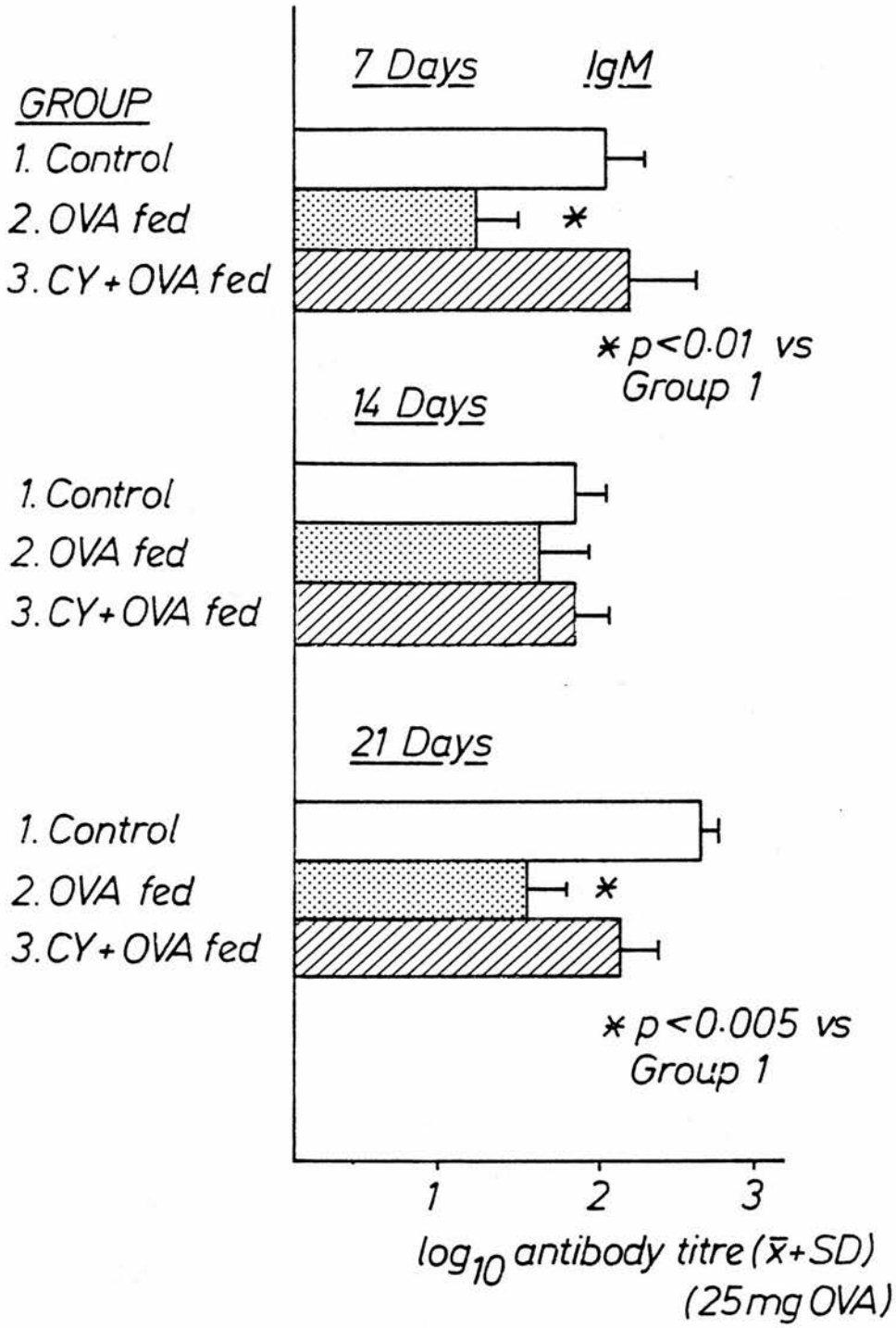
In contrast to the results after 14 days, there was no suppression of antibody titres detectable in either group after three weeks (Figure 4.8) and mercaptoethanol resistant antibodies accounted for the majority of antibodies detected.

A different picture was obtained when the animals were fed 25 mg ovalbumin (Figure 4.9). Although there were no differences in antibody titres after 14 days, the response of ovalbumin fed mice was suppressed (93% suppression, $p<0.005$) after three weeks. Cyclophosphamide pretreatment partly reversed the suppressive effect of the 25 mg ovalbumin feed (67% suppression, pNS) and these titres were not different from control but were significantly higher than in tolerant animals ($p<0.05$) (Figure 4.9).

As judged by the evaluation of mercaptoethanol sensitive antibody responses, animals fed 2 mg ovalbumin had their peak response after two weeks, and at one week when they were fed 25 mg ovalbumin. At these times, both doses of oral antigen suppressed the subsequent IgM (mercaptoethanol sensitive) response compared to controls, 93% suppression in 25 mg ovalbumin fed animals, ($p<0.01$) and 60% suppression in 2 mg ovalbumin fed mice ($p<0.05$).

Since these antibody titres have been obtained by calculating a ratio between total antibody titres and mercaptoethanol resistant antibody titres and were not measured specifically, the pattern of the IgM response need to be cautiously interpreted.

On the whole, the results indicate that IgM responses of mice may be tolerised even by low doses of oral protein while larger amounts are required to achieve suppression of the IgG response. The tolerance of



4.9 **Immunomodulation of oral tolerance by pretreatment with cyclophosphamide**

Humoral antibody responses in mice which were fed 25 mg ovalbumin two days after injection with cyclophosphamide (100 mg/kg). Seven days after immunisation, 2-mercaptoethanol sensitive (IgM) antibody levels were suppressed (group 2) and this suppression was abrogated by cyclophosphamide (group 3). This effect was not detectable after 14 days. After 21 days IgG antibody responses were suppressed in group 2 ($p<0.005$ vs. group 1, $p<0.05$ vs. group 3). Cyclophosphamide treatment partially reversed the suppression of these antibody responses observed in group 2.

IgM responses appeared to be more susceptible to abrogation by cyclophosphamide pretreatment, although there was great variation between individual groups.

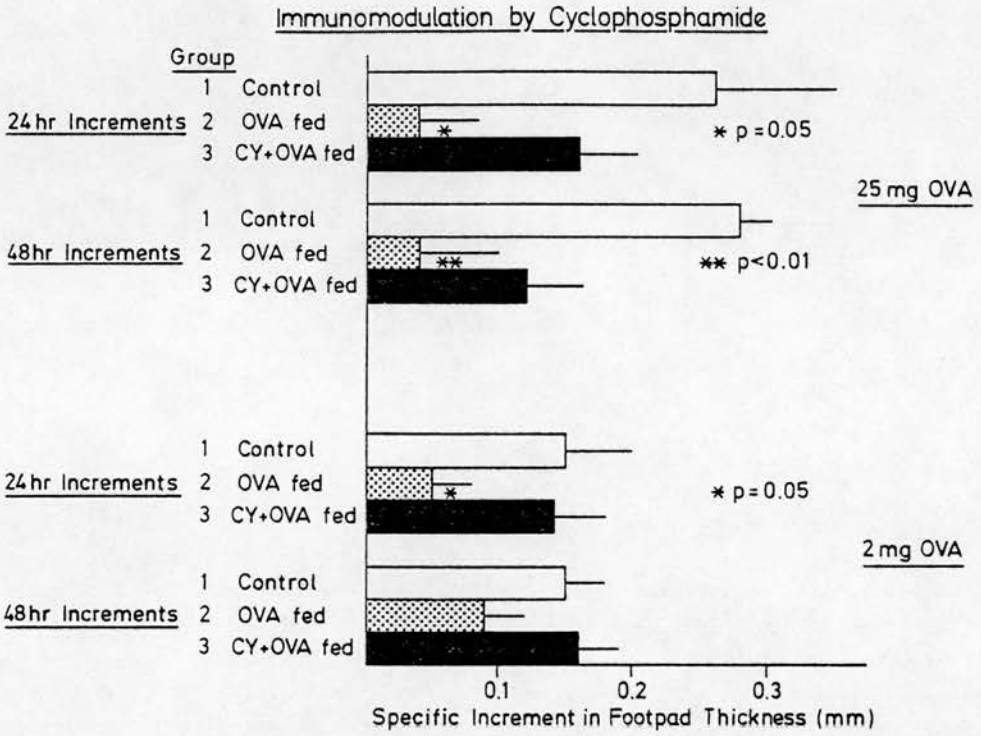
2. Cell mediated immune responses

Figure 4.10 shows the CMI responses in mice fed 2 or 25 mg ovalbumin which was assessed three weeks after immunisation by measuring the specific increase in footpad thickness at 24 hours. The results illustrate that both doses of oral ovalbumin will suppress a subsequent CMI response, with 88% suppression ($p<0.05$) in 25 mg ovalbumin fed mice and 64% suppression in the mice fed 2 mg ovalbumin ($p<0.05$) compared to controls. Cyclophosphamide given to mice fed 25 mg ovalbumin returns their systemic CMI responses to a value which is midway between the control and tolerant mice and which is not significantly different from either group. It is important to note, however, that the mice receiving cyclophosphamide before feeding 2 mg of ovalbumin have completely normal CMI responses and show no residual tolerance. Similar results were obtained 48 hours later Figure 4.10, confirming the delayed type hypersensitivity nature of the reactions measured and the consistency of the tolerant state. Thus, feeding 2 and 25 mg ovalbumin suppress the CMI responses significantly.

II. **Comment**

The results presented here confirm that when the first encounter with an antigen is by the oral route, a range of different effects on the systemic immune response may be observed.

Depending on the dose of oral protein used, I have shown that



4.10 **Immunomodulation of oral tolerance by pretreatment with cyclophosphamide**

Delayed type hypersensitivity responses in mice which were fed 2 or 25 mg ovalbumin two days after injection with cyclophosphamide (100 mg/kg). Abrogation of tolerance for CMI was complete in mice which received 2 mg ovalbumin (group 2). The difference in specific footpad increments was maintained after 48 hours.

feeding 25 mg ovalbumin two weeks before immunisation will reduce humoral and CMI responses to ovalbumin. Mice which were fed 2 mg ovalbumin only showed a suppression of their CMI responses and IgM responses, whereas IgG antibody levels were unaffected.

Cyclophosphamide partially reversed tolerance induced by feeding 25 mg ovalbumin and completely abrogated the tolerance for CMI found after feeding 2 mg ovalbumin. The results indicate that systemic CMI is readily tolerized and that it is completely abrogated by cyclophosphamide, a finding in line with the theory that tolerance to orally presented antigen is dependent on the induction of a cyclophosphamide sensitive suppressor cell system.

The dose dependent suppression of systemic CMI and antibody responses and the differential effects of cyclophosphamide treatment suggest that oral tolerance is dependent on several immunoregulatory factors including T-suppressor cells. Whether these effects are strain dependent or due to the action of this drug on the immune system alone will be considered in the next section.

C. EFFECTS OF CYCLOPHOSPHAMIDE ON SYSTEMIC TOLERANCE IN DIFFERENT STRAINS OF MICE

Having demonstrated that cyclophosphamide given intraperitoneally two days before antigen feeding, partially reverses the tolerance for humoral immunity and abrogates CMI tolerance (see previous section) it was of special interest whether the effect of cyclophosphamide is specific to BALB/c mice only. Since my original experiments were done in BALB/c mice and most of the relevant work on oral tolerance was conducted in BDF_1

mice, I compared the effects of this drug on systemic immune responses in these two strains before examining the effects of cyclophosphamide on induction of local intestinal CMI (see below).

I. Experiments and results

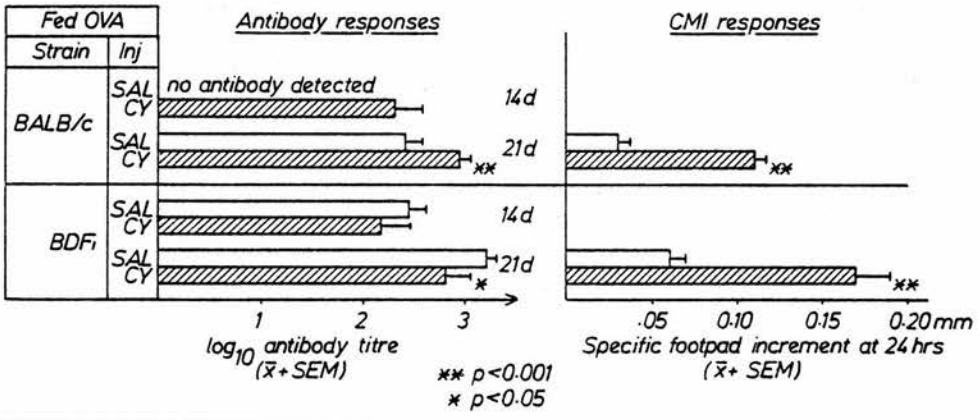
The general experimental protocol (Figure 4.3) was followed and closely age matched females were injected intraperitoneally with saline or 100 mg cyclophosphamide/kg body weight and fed 20 mg ovalbumin two days later.

1. Antibody responses two and three weeks after immunisation

BALB/c mice injected with saline showed no detectable antibody production after 14 days compared to a titre of 2.3 $\pm$ 0.32 ($\bar{x}$ $\pm$ SD) in the cyclophosphamide treated group. This obvious trend of reversing tolerance by cyclophosphamide was maintained after three weeks where the tolerant group reached only 33% (antibody titre 2.4 $\pm$ .17) of the antibody responses of the cyclophosphamide treated group (antibody titre 2.92 $\pm$ 0.13, $p<0.001$). These results confirm that cyclophosphamide reverses the humoral tolerance seen after ovalbumin feeding (Figure 4.11).

BDF_1 mice showed a different pattern which is demonstrated two and three weeks after immunisation. Two weeks after immunisation, the saline injected (tolerant) mice developed a titre of 2.45 $\pm$ .23 compared with 2.18 $\pm$ .34 in the cyclophosphamide treated group. This trend was maintained after three weeks where the saline injected group exhibited a titre of 3.22 $\pm$ 0.13 and the cyclophosphamide group again showed a considerable lower antibody titre (2.84 $\pm$ 0.31, $\bar{x}$ $\pm$ SEM $p<0.05$ (Figure 4.11).

Modulation of Oral Tolerance by Cyclophosphamide
: Effects of CY pretreatment in different mouse strains



4.11 Modulation of oral tolerance by pretreatment with cyclophosphamide: Effects of cyclophosphamide in different mouse strains

BALB/c and BDF_1 mice were injected with cyclophosphamide, fed ovalbumin and treated according to the general protocol (4.3).

Antibody responses: Cyclophosphamide reversed the immunosuppression observed after ovalbumin feeding in BALB/c animals as previously shown in Figure 4.9 ($p<0.001$), whereas the immune suppression in BDF_1 animals was further increased ($p<0.05$).

CMI responses: Both strains exhibited a similar reversal of immune suppression after cyclophosphamide treatment ($p<0.001$).

2. Cell mediated immune responses three weeks after immunisation

BALB/c mice: Cyclophosphamide had a profound effect on CMI reactions and abrogated the suppression of delayed type hypersensitivity responses seen after ovalbumin feeding (0.03 $\pm$ 0.007 mm vs. 0.11 $\pm$ 0.004 mm; $\bar{x} \pm$ SEM, $p<0.001$; Figure 4.11). The results confirmed that cyclophosphamide abrogates humoral and CMI tolerance in BALB/c mice.

BDF_1 mice: As seen in Figure 4.11, BDF_1 mice responded to cyclophosphamide treatment in the same way as did BALB/c mice; the drug abrogated tolerance for CMI (0.06 $\pm$ 0.01 mm vs. 0.17 $\pm$ 0.02 mm; $\bar{x} \pm$ SEM, $p<0.001$).

II. **Comment**

An unexpected finding was the demonstration of a difference in susceptibility to cyclophosphamide treatment in the two strains of mice tested. This difference affected only the humoral immune responses.

The experiments have been repeated with virtually identical results by myself and independently by M Pickering (personal communication 1982) and are in line with published reports (Hanson & Miller 1981). The reason for this different susceptibility to cyclophosphamide treatment cannot be given and the results presented are not due to the experimental design. It seems however possible that the differences in susceptibility to cyclophosphamide are genetically determined and evidence along these lines will be discussed later (General Discussion).

D. EFFECTS OF CYCLOPHOSPHAMIDE ON SYSTEMIC IMMUNITY

Although cyclophosphamide is rapidly cleared from the circulation (Tew and Taylor 1977), it has been found to increase the systemic

immune responses at a later date. It was therefore important to investigate the effects of cyclophosphamide on systemic immunity at the time of parenteral immunisation.

I. Experimental protocol and results

In line with the time scale of other experiments, BALB/c mice were given 100 mg of cyclophosphamide 16 and nine days before immunisation, whereas BDF_1 mice received cyclophosphamide nine days before immunisation.

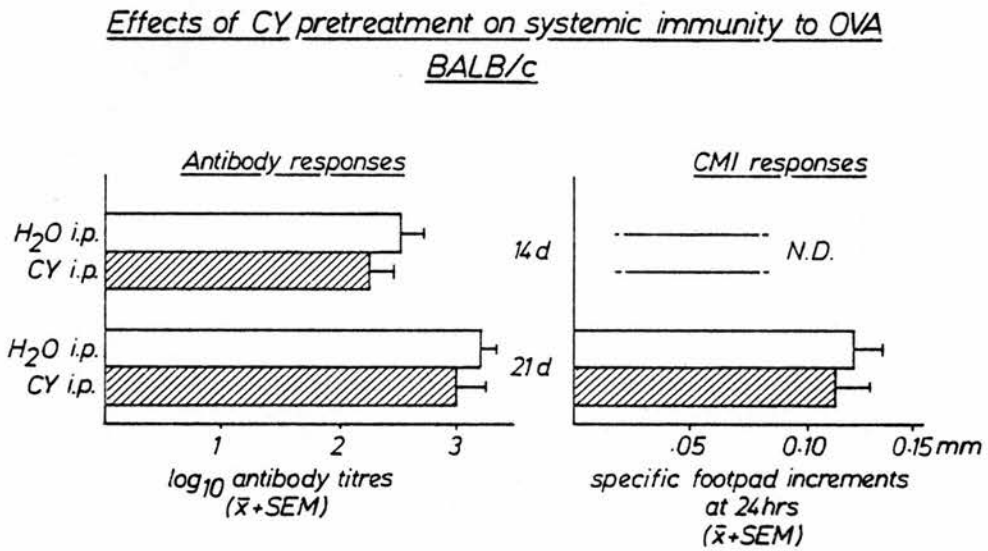
1. Humoral antibody and cell mediated responses

Serum antibodies in BALB/c animals immunised 16 days after cyclophosphamide were measured by passive haemagglutination. Figure 4.12 shows that cyclophosphamide given 16 days before immunisation had no significant effect on either antibody or CMI responses, although the humoral responses were slightly suppressed. These minor effects were consistent, but not significant and observed in the BALB/c and BDF_1 animals (Figure 4.13) which were immunised nine days after cyclophosphamide treatment.

It seems, therefore, highly unlikely that the reversal of oral tolerance by cyclophosphamide could be explained by a stimulatory action on the immune system.

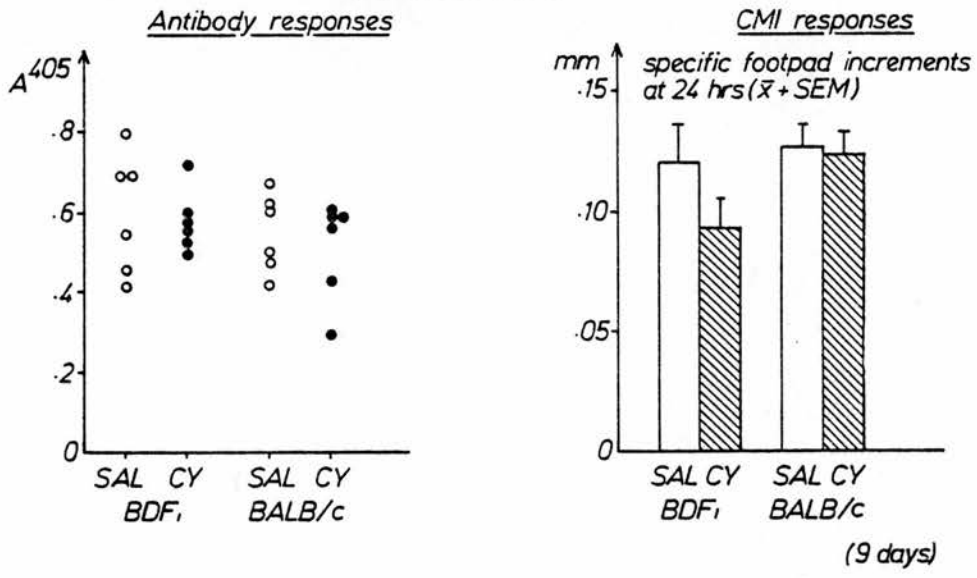
II. Comment

Systemic immune responses in animals which were injected with cyclophosphamide 16 or nine days before immunisation with ovalbumin suggested that the immune enhancement seen after cyclophosphamide treatment prior to oral antigen is not dependent on stimulatory effects on systemic immunity. The results obtained in this series of



4.12 **Effects of cyclophosphamide on systemic immunity**

Cyclophosphamide had no effect on antibody or CMI responses if given 16 days before immunisation. Note that systemic responses were slightly, but not significantly, suppressed.



4.13 **Effects of cyclophosphamide on systemic immunity** (ELISA)

Cyclophosphamide had no significant effect on serum antibody or CMI responses when given nine days before immunisation. Note that there is a slight, but not significant, reduction of systemic immunity in both strains of mice (BALB/c, BDF_1) (Compare Figure 4.12).

experiments confirm that oral tolerance is abrogated by cyclophosphamide given two days before oral antigen administration. Divergent effects effects on the humoral antibody production in different inbred strains of mice strengthen the theory that humoral immunity and CMI are controlled by different - not necessarily exclusive - homeostatic mechanisms involving T-suppressor cells. Furthermore, after demonstration of these effects, it seems important to take care even in extrapolating results from one inbred mouse system to another without carefully assessing the properties of the system in use.

The above findings indicate that comparable states of tolerance to oral antigen may be controlled by different homeostatic mechanisms. Phenotypically similar degrees of immunological tolerance have been observed in animals in which the tolerant state was due to clonal deletion or active suppression by T-suppressor cells (see: Introduction). It is conceivable that these two strains of mice differ in certain immunoregulatory circuits but exhibit phenotypically identical humoral and CMI responses after oral ovalbumin presentation.

The exact nature of these phenomena remains to be established and is beyond the scope of this thesis.

E. MODULATION OF ORAL TOLERANCE TO OVALBUMIN BY A GRAFT-VERSUS-HOST-REACTION (GvHR)

Mice experiencing a GvHR develop a depression of systemic humoral and CMI responses to sheep erythrocytes (Treiber & Lapp 1976). Although some lymphoid cells are destroyed during a GvHR, those immunosuppressed mice still possess antigen reactive cells (Treiber & Lapp 1973) and they can mount a delayed type hypersensitivity reaction

to different antigens in complete Freund's adjuvant without making an antibody response. The hypothesis for the underlying mechanism implies that a population of adherent cells and/or their products (monokines) exerts a regulatory role on T-helper and T-DTH effector cells (Treiber & Lapp 1976).

It has also been demonstrated that systemic immunity is enhanced in the early, initial phase of a GvHR (Davis, Cole & Schaffer 1970). Because of the profound and well documented immunoregulatory disturbances in these animals and the experience already gathered with this animal model in the laboratory (Mowat & Ferguson 1982), I decided to investigate the effects of a low grade GvHR on the induction of tolerance to ovalbumin.

By using this animal model of a GvHR which does not induce mucosal damage, it was feasible to modulate and to investigate the immune responsiveness after an antigen feed. The important question whether the modulation of systemic immunity to an antigen may also induce a local CMI on subsequent antigen challenge will be discussed later (Chapter 7).

In this section I shall state the relevant findings used as indicators for assessment of a GvHR and shall discuss mainly the effects of a low grade GvHR on modulation of oral tolerance.

I. Experiments and results

GvHR was induced by intraperitoneal injection of 6×10^7 CBA spleen cells suspended in RPMI 1640 into (CBA x BALB/c) F_1 males and females aged 6-8 weeks. Controls were injected with medium alone. On day 12 after induction of the GvHR, control animals and mice with a GvHR

were fed 25 mg ovalbumin or saline and treated according to the general experimental protocol (Figure 4.3). To assess morphological parameters and whether the GvHR induction was successful in these animals, further groups of mice, which had received spleen cells or medium on the same day, were sacrificed 12 and 40 days after GvHR. The animals were weighed, pieces of jejunum taken for histological examination and the Spleen Index was calculated after Simonsen (1962).

1. Indicators of a graft-versus-host reaction

All animals remained healthy throughout the experiments. Groups of animals which were sacrificed 12 or 40 days after GvHR induction showed a Spleen Index of 2.36 (12 days) and 1.59 (40 days) indicating substantial splenic hypertrophy.

2. Intraepithelial lymphocyte infiltration

Intraepithelial lymphocyte infiltration counts were increased in animals with a GvHR (18.2 ± 1.1 vs. 10.2 ± 0.1, $p<0.001$) after 40 days. These parameters taken together (see also Chapter 7) were taken as evidence that a low grade GvHR had been induced in these mice.

II. Effects of graft-versus-host reaction on oral tolerance

Assessment of the parameters described above revealed that the animals which showed no signs of disease did have a low grade GvHR and that changes in the immune responsiveness could reasonably be attributed to the immunoregulatory disturbance induced by a GvHR.

1. Experimental protocol and results

Mice with GvHR and sham injected controls were fed either saline

or 25 mg ovalbumin 12 days after the initial treatment. Systemic immunoresponses were assessed three weeks after immunisation with ovalbumin in complete Freund's adjuvant (as outlined in the experimental protocol (Figure 4.3)).

(a) Humoral antibody responses

Antibody responses were assessed in mice with or without GvHR which had been fed ovalbumin. Control animals fed ovalbumin showed a marked suppression of their antibody responses (2.35 ± 0.08 vs. 3.08 ± 0.15, $\bar{x} \pm$ SD, 80% suppression, $p<0.05$). Animals with GvHR exhibited only 46% suppression three weeks after immunisation (3.05 ± 0.24 vs. 3.32 ± 0.16, pNS) indicating a partial reversal of the tolerisability during the early phase of a GvHR (Figure 4.14). It is notable that animals with GvHR exhibited a consistently higher humoral response than their normal counterparts.

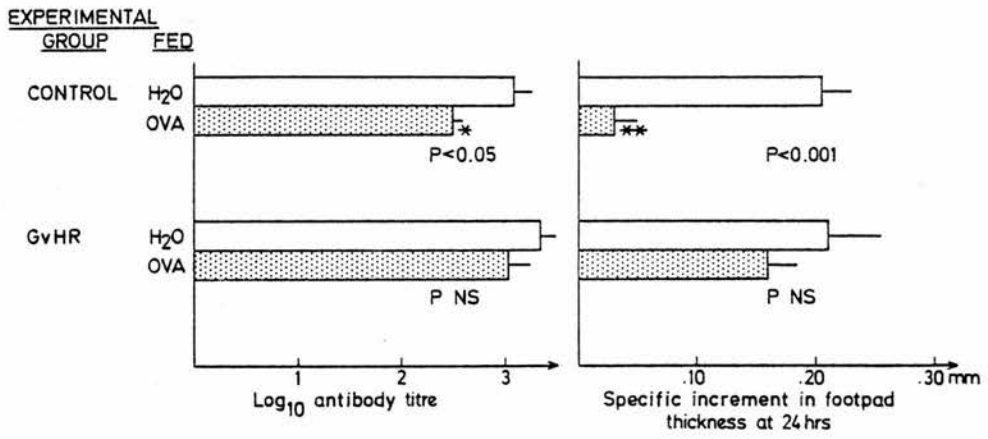
(b) Cell mediated immune responses

The CMI responses were affected in a similar way and animals with GvHR fed ovalbumin showed only 24% reduction of their footpad swelling 24 hours after challenge (0.21 ± 0.02 mm vs. 0.16 ± 0.03 mm, pNS) whereas control animals exhibited 85% suppression (0.20 ± 0.02 mm vs. 0.03 ± 0.01 mm, $p<0.001$, $\bar{x} \pm$ SEM) which was the usual degree of suppression (Figure 4.14).

III. Effects of a graft-versus-host response on systemic immunity

The above results show that animals fed on day 12 of a GvHR were not tolerised by the usual feeding regimen. The relevant measurements were made at 40 days after induction of GvHR. To gain more insight into the underlying disturbances in immunoregulation in these animals,

EFFECTS OF GvHR ON TOLERANCE TO FED ANTIGEN (DAY 12)
(3 weeks after immunisation with OVA in CFA)



4.14 Effects of a graft-versus-host response on the induction of oral tolerance

Serum antibody and CMI responses three weeks after immunisation. Feeding ovalbumin 12 days after induction of GvHR did not lead to systemic tolerance.

which led to the partial reversal of oral tolerance, the immuno-responsiveness on the 12th day after GvHR induction was investigated.

1. Experimental protocol and results

Male and female animals with GvHR and control mice injected with medium alone were immunised on the 12th day of the GvHR with 100 µg ovalbumin in complete Freund's adjuvant and humoral immunity and CMI assessed two and three weeks after immunisation.

(a) Effects of a graft-versus-host reaction on humoral immunity

Mice with GvHR all responded with an increased antibody production two and three weeks after immunisation. The antibody titre in GvHR animals two and three weeks after immunisation was 2.54 ± 0.2 and 3.66 ± 0.19 compared to 2.0 ± 0.5 and 2.95 ± 0.36 in intact animals (two weeks $p<0.02$, three weeks $p<0.001$, $n = 17$, $\bar{x} \pm SD$) (Figure 4.15).

(b) Effects of a graft-versus-host reaction on cell mediated immune responses

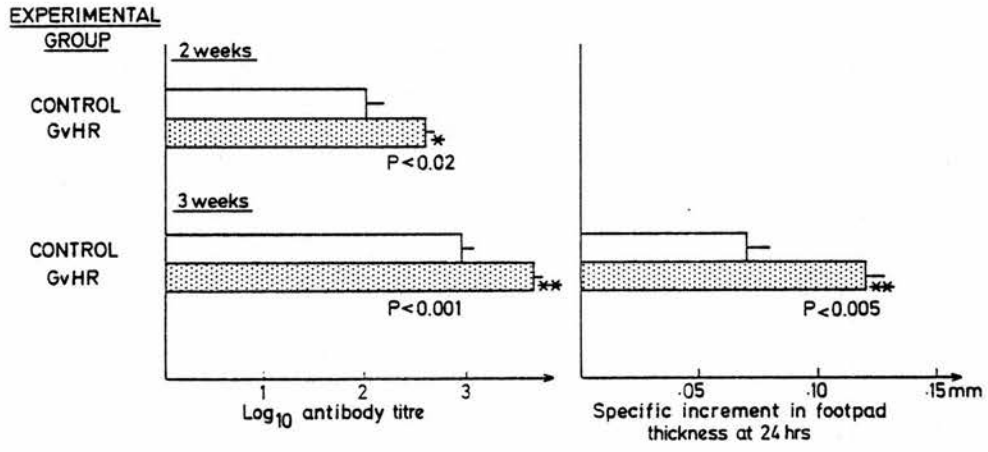
CMI immunity was enhanced in a similar way. Control animals showed a footpad swelling of 0.07 ± 0.01 mm, whereas the responses were increased to 0.12 ± 0.01 mm ($p<0.005$, 77% increase, $\bar{x} \pm SEM$) in mice with GvHR (Figure 4.15) and this difference was maintained after 48 hours (80% increase, $p<0.05$) proving the delayed type hypersensitivity nature of this response.

IV. **Comment**

Injection of parental spleen cells into unirradiated F_1 hybrids led to a low grade GvHR as assessed by the increased Spleen Index and mucosal infiltration of intraepithelial lymphocytes.

The GvHR did not lead to overt disease and the animals remained

EFFECTS OF GvHR (DAY 12) ON SYSTEMIC IMMUNE RESPONSE TO OVA



4.15 **Effects of a graft-versus-host response on systemic immunity**

Mice with a GvHR were immunised with ovalbumin and systemic humoral and CMI responses measured two and three weeks later. Humoral antibody responses were significantly enhanced at two ($p<0.02$) and three weeks ($p<0.001$). Specific footpad increments at three weeks were also enhanced ($p<0.005$).

healthy throughout the experiment. The GvHR had, however, pronounced effects on the host immune system. Tolerance after feeding ovalbumin was partially reversed. This was most probably due to the immunostimulatory effects of GvHR since animals immunised 12 days after induction of a GvHR all showed an enhanced systemic immune response. This could have been initiated by adherent cell dependent activation of T-helper/amplifier cells during a GvHR as suggested by Treiber and Lapp (1976), although the present study was not designed to investigate these mechanisms.

F. MODULATION OF ORAL TOLERANCE TO OVALBUMIN BY MURAMYL-DIPEPTIDE

Immunostimulatory treatment with the synthetic adjuvant N-acetyl muramyl-dipeptide has been shown to enhance and suppress systemic immune responses. The timing of antigen administration in relation to the adjuvant injection seems to be of crucial importance (Leclerc, Juy, Bourgeois & Chedid 1979). Muramyl-dipeptide is the simplest structural unit of bacterial peptidoglycans which is capable of replacing whole killed bacteria in Freund's complete adjuvant (Chedid, Parant, Parant, Lefrancier, Choay & Lederer 1977). The primary action seems to be on macrophages and if administered in close temporal association with antigen it also has a stimulatory effect on T-helper cells (Leclerc et al 1979). As do many other adjuvants, it stimulates macrophages and the production and liberation of monokines which activate B and T-cells (Fevrier, Birrien, Leclerc, Chedid & Liacopoulos 1978, Pabst & Johnston 1980).

Since an adherent cell population seems to have been responsible for the reversal of tolerance during a GvHR, I decided to use muramyl-

dipeptide as an immunomodulatory agent because its mode of action is well defined (Löwy, Bona & Chedid 1977, Leclerc et al 1979, Dukor, Tarcsay & Baschang 1979) and to get additional information on the role of adherent cells and T-helper cells during the reversal of tolerance to ovalbumin.

I. Experimental and protocol results

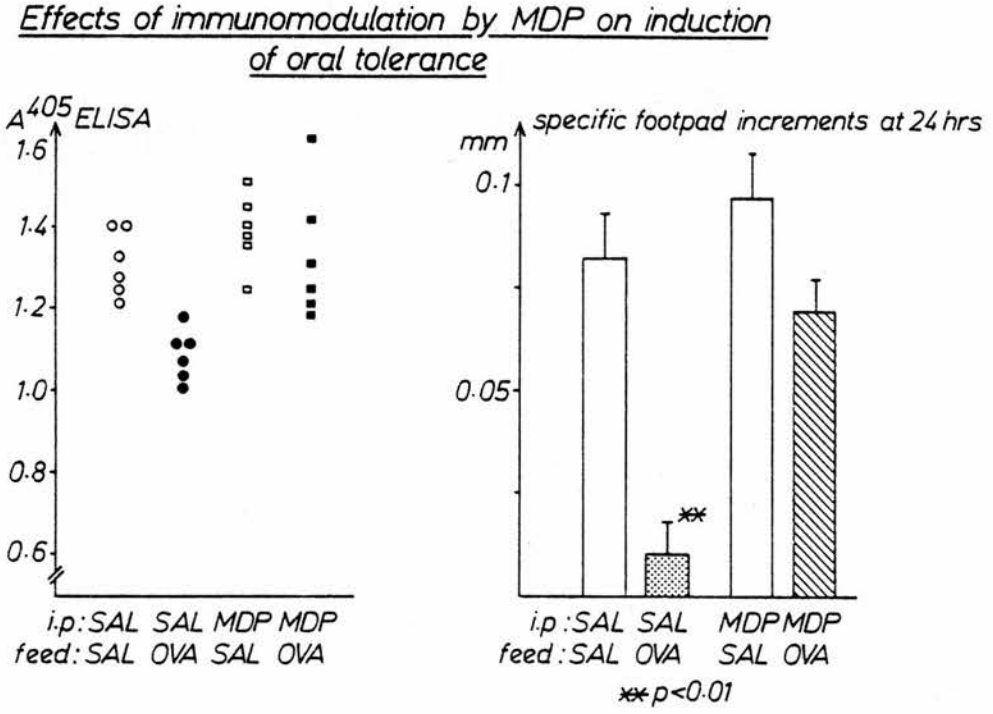
Following the standard experimental designs, animals were injected saline or 50 μg muramyl-dipeptide immediately before one half of each group was fed saline or ovalbumin. After this initial treatment, all mice were immunised and the systemic immunity tested as outlined in the general experimental protocol. In this way, not only the effects of muramyl-dipeptide on the induction of oral tolerance were investigated, but also the effects on systemic immunity.

1. Humoral antibody responses

Animals injected saline and fed either saline or ovalbumin demonstrated the anticipated pattern; ovalbumin fed animals showed a significant suppression of their responses ($p<0.01$) compared to their controls (Figure 4.16). Injection of muramyl-dipeptide abrogated systemic tolerance, non-fed and ovalbumin fed animals showing a similar antibody response. On the other hand, the adjuvant injected on its own did not increase the antibody responses in the saline fed control group (Figure 4.16).

2. Cell mediated immune responses

A similar pattern was found for CMI. The saline injected ovalbumin fed group demonstrated 88% suppression (0.082 ± 0.01 vs.



4.16 Effects of muramyl-dipeptide on the induction of oral tolerance

Mice were injected with muramyl-dipeptide before they were fed saline or ovalbumin. All groups were immunised seven days later and treated according to the general protocol (4.3). Muramyl-dipeptide abrogated the suppression of serum antibody responses. Tolerance for CMI responses was also reversed.

0.01 ± 0.008 mm, $p<0.001$, $\bar{x}$ ± SEM), whereas the delayed type hypersensitivity responses of animals injected muramyl-dipeptide before feeding ovalbumin were not significantly (30%) suppressed (0.097 ± 0.012 mm vs. 0.068 ± 0.008 mm, pNS, $\bar{x}$ ± SEM) (Figure 4.16).

II. **Comment**

N-acetyl-muramyl-dipeptide in saline given at the same time as the antigen abrogates oral tolerance for humoral and cell mediated immunity, although the abrogation for CMI was not complete. These effects are likely to be due to macrophage activation and altered antigen presentation and/or handling by immunoregulatory cells. Local intestinal or mucosal factors, such as histological damage are not likely to be of importance in this respect (see Chapter 7).

G. INDUCTION OF "ORAL TOLERANCE" BY COLONIC ADMINISTRATION OF OVALBUMIN

I. **Questions to be answered**

These experiments were performed to investigate two main questions:

1. Are Peyer's patches as specialised and organised lymphoid tissues necessary for the induction of tolerance after oral administration?
2. What is the role of intraluminal digestion in induction of tolerance to protein antigens?

The aboral route of administering drugs, for example, by suppositories, is frequently used in the paediatric age group and pharmacological effects are obvious, although the absorption is variable. In radiolabelling and antigen-binding studies, it was shown

that rectally administered heterologous gamma globulin is absorbed in significant amounts and that a sizeable proportion of it retains its antigen-binding activity (Nagatomi, Ogita, Okudaira & Mitzushima 1980). Since uptake of immunoreactive ovalbumin after rectal administration into the circulation was likely to occur, I decided to investigate the uptake after oral and colonic administration. This would enable me to discuss any differences in tolerance to ovalbumin in the light of these findings.

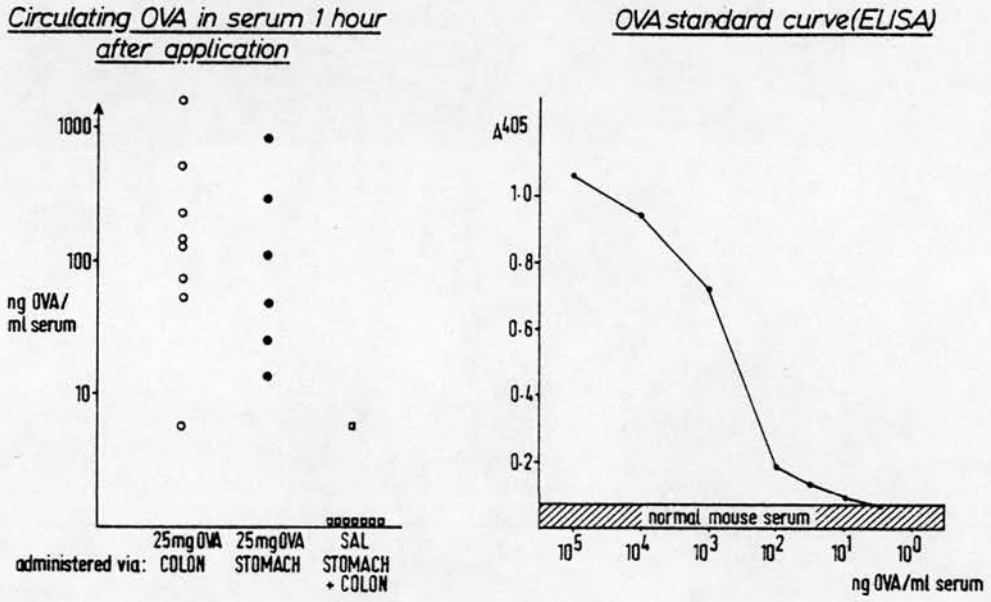
II. Experimental protocol results

Experiments were conducted in BDF_1 mice according to the general experimental protocol (Figure 4.3) and included orally dosed control groups. Six out of 16 mice in each group were bled out after 60 minutes and the circulating concentrations of ovalbumin measured with a direct enzyme immunoassay (see Technical developments, Chapter 3).

1. Circulating ovalbumin after oral or colonic administration

The circulating amounts of ovalbumin after one hour varied considerably in both treatment groups. The mean amount of ovalbumin detected in the 'colon' group was 288.0 $\pm$ 134 ng/ml serum (range 6 - 1122 ng/ml, mean = 0.0015%) and in the 'stomach' group 212.0 $\pm$ 124 ng/ml (range 13 - 795 ng/ml, mean = 0.00085%) (Figure 4.17).

The detection threshhold of the ELISA test system was 3ng/ml (Figure 4.17). There were no significant differences and the results indicate that colonic administration was as effective as judged by the amount of circulating antigen. If there was any leakage of antigen out of the rectum due to defecation, it was not significant enough to affect the overall uptake in comparison to the oral administration.



4.17 Circulating ovalbumin in the serum after intragastric and colonic administration

The circulating amounts of ovalbumin in the serum 60 minutes after administration of 25 mg ovalbumin via the 'colon' or 'stomach' are shown on the left hand side of the graph. The corresponding ovalbumin standard curve (ELISA) is shown on the right. The detection threshold was 3 ng ovalbumin/ml serum and the hatched area indicates the mean (+ 2SD) absorbance readings of normal mouse serum.

2. Effects of feeding a concentrated ovalbumin solution on systemic immunity

The concentration of the standard feeding solution was 100 mg/ml and the following experiment was performed to establish whether a more concentrated ovalbumin solution would influence the degree of tolerance induced by feeding. Oral administration of 25 mg ovalbumin applied in a concentrated ovalbumin solution (400 mg/ml) in 0.065 ml induced the same degree of suppression for humoral immunity and CMI as observed when the same dose of ovalbumin was fed in a 100 mg/ml solution. Suppression of antibody responses was 80% (3.1 ± 0.21 vs. 2.42 ± 0.21, $p<0.01$, $\bar{x} \pm SD$) and of CMI responses, 86% (0.12 ± 0.01 mm vs. 0.017 ± 0.01 mm, $p<0.01$, $\bar{x} \pm SEM$) (Figure 4.18, 'Stomach'group)

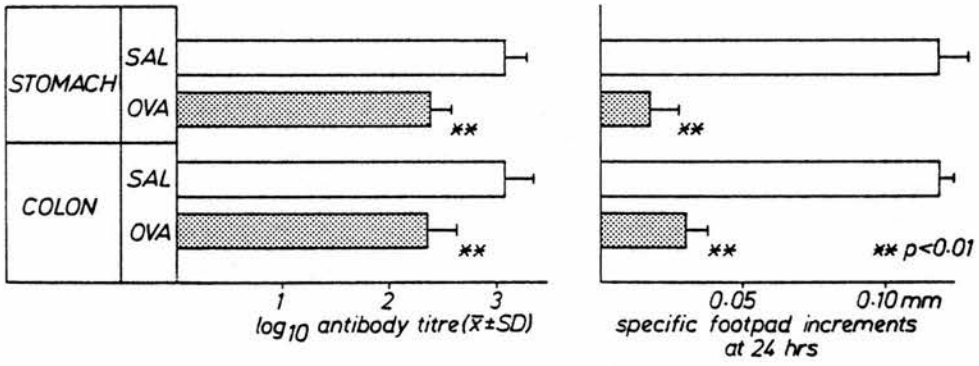
3. Effects of colonic administration of ovalbumin on systemic immunity

Animals receiving identical amounts of ovalbumin exhibited profound tolerance for both antibody and CMI responses. The antibody responses showed a 81% suppression compared to animals which received a saline enema (3.11 ± 0.28 vs. 2.38 ± 0.27, $p<0.01$, $\bar{x} \pm SD$). CMI responses were suppressed by 75% (0.12 ± 0.004 mm vs. 0.03 ± 0.009 mm, $p<0.01$, $\bar{x} \pm SEM$) (Figure 4.18, 'Colon' group).

III. Comment

A solid and profound state of tolerance was induced by colonic administration of antigen. Surprisingly, the uptake of ovalbumin after colonic administration was similar to that observed after oral dosing. The tolerance for CMI was slightly less profound than that observed after feeding ovalbumin. Oral contact with ovalbumin contaminated

Effects of colonic or intragastric administration of OVA on systemic immunity



4.18 Effects of colonic or intragastric administration of ovalbumin on systemic immunity

Different groups of mice were given saline or ovalbumin via stomach or colon seven days before they were immunised and treated according to the general protocol (4.3). Systemic hyporesponsiveness was induced after ovalbumin administration in both groups.

faeces after rectal administration cannot be completely excluded. However, it seems unlikely that this route of antigen exposure could have accounted for the observed results. These results indicate that intraluminal digestion or antigen access via Peyer's patches is not necessary for the induction of systemic tolerance after enteral antigen administration. Small lymphoid aggregates scattered throughout the intestinal tract seem to be effective in initiating the hyporesponsiveness after antigen feeding. Furthermore presentation of gut processed antigen via the circulation to the gut associated lymphoid tissues may be sufficient to create a hyporesponsive state.

Chapter 5

THE ROLE OF THE GUT IN GENERATING IMMUNOLOGICAL HYPORESPONSIVENESS

As demonstrated earlier, oral tolerance to ovalbumin could be modulated by various means (cyclophosphamide, GvHR, muramyl-dipeptide). These treatments resulted mainly in total or partial abrogation of tolerance. In those experiments where cyclophosphamide has been shown to abrogate oral tolerance, two main features could have been accountable for the reversal: 1) the action on rapidly dividing T-suppressor cell precursors or 2) the effects on the gut epithelium. Effects of cyclophosphamide on the gut have been well documented (Ecknauer & Löhrs 1976, Mowat 1981). These , although minor, mucosal changes on one hand could have led to the alteration of antigen uptake and to a change in antigen processing. Interference with these important steps may have led to disturb the balance between the amounts of immunogenic or tolerogenic protein moieties present after feeding. Possible effects on either T-cells or intestinal mucosa were investigated by adoptive transfer of serum which was recovered 60 minutes after feeding of ovalbumin.

In a further series of experiments, the impacts of a severe intestinal damage and subsequent immunosuppression were investigated. Irradiation (1000 rad) was used as a tool to modulate the immunoresponsiveness and to induce intestinal damage of the donor mice before feeding ovalbumin and retrieving the serum for transfer. In doing so, it was expected to investigate whether the mucosal changes after irradiation could lead to qualitative and quantitative differences of circulating antigen. By transfer of serum from irradiated mice, one would thus gain more insight into the role of the gut wall in inducing tolerance to protein antigens.

A. TRANSFER OF TOLERANCE BY SERUM OF OVALBUMIN FED DONORS AND THE EFFECTS OF CYCLOPHOSPHAMIDE

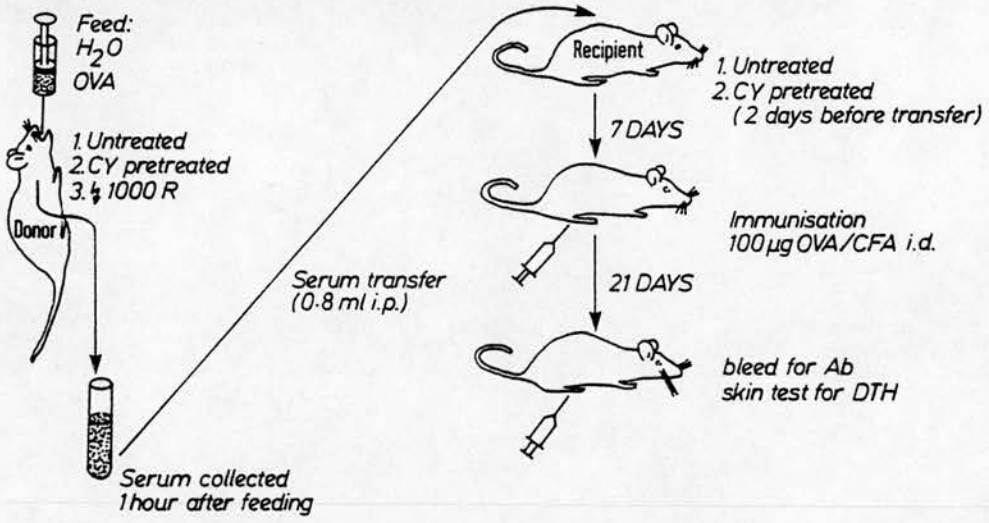
I reasoned that it should be feasible to examine the effects of "gut processsing" on the immunogenicity or tolerogenicity of fed antigen by comparing the in vivo effects of antigen which had been absorbed across the gut into the serum of mice, with the effects of administration of similar amounts of native antigen. A serum transfer protocol was used in which serum collected one hour after feeding mice ovalbumin was transferred intraperitoneally into syngeneic recipients (Figure 5.1). The immunological properties (tolerogenic or immunogenic) of the antigen in the transferred serum were then assessed by measurements of humoral and CMI responses in the recipients after a subsequent parenteral injection of ovalbumin. I also investigated the site of action of cyclophosphamide in modulating oral tolerance by separately treating donors and recipients of serum with cyclophosphamide (Figure 5.1). If it acts at the level of the intestine, pretreatment of donors should alter immune responses in recipients. If cyclophosphamide exerts its effect by eliminating suppressor cells in recipients, this treatment should then alter their immune responses.

I. Experimental protocol and results

The basic immunological protocol is illustrated in Figure 5.1 and the individual treatment groups are listed in Table 5.2.

1. Serum antibodies in recipients before parenteral immunisation

All recipient mice were bled immediately prior to parenteral immunisation with ovalbumin in complete Freund's adjuvant. None of the



5.1 **Schematic diagram of serum transfer experiments**

Saline or ovalbumin was fed to untreated or pretreated donors as indicated and their sera were collected one hour after feeding. 0.8 ml of serum were then transferred (i.p.) into untreated or cyclophosphamide pretreated recipients which were immunised seven days later.

Table 5.2

Details of experimental protocol and treatment of groups

Recipient mice groups	Treatment of serum donors		Treatment of recipients		
	Day -2 (intraperitoneal)	Day 0 (feed)	Day -2 (intraperitoneal)	Day 0	Day 7 - 28
A	H_2O	H_2O	H_2O	serum i.p.	all groups
B	H_2O	OVA	H_2O	serum i.p.	
C	CY	H_2O	H_2O	serum i.p.	Day 7
D	CY	OVA	H_2O	serum i.p.	100 µg OVA i.d.
E	H_2O	H_2O	CY	serum i.p.	via footpad
F	H_2O	OVA	CY	serum i.p.	Day 21 and
G	-	-	-	saline i.v.	Day 27 - bleed
H	-	-	-	1 µg OVA i.v.	for antibodies
I	-	-	-	10 µg OVA i.v.	Day 28 - skin
J	-	-	-	100 µg OVA i.v.	test for DTH
K	-	-	-	10 µg OVA i.p.	
L	-	-	-	100 µg OVA i.p.	

sera contained detectable antibodies against ovalbumin.

2. Effects of serum transfer on systemic immune responses of recipients

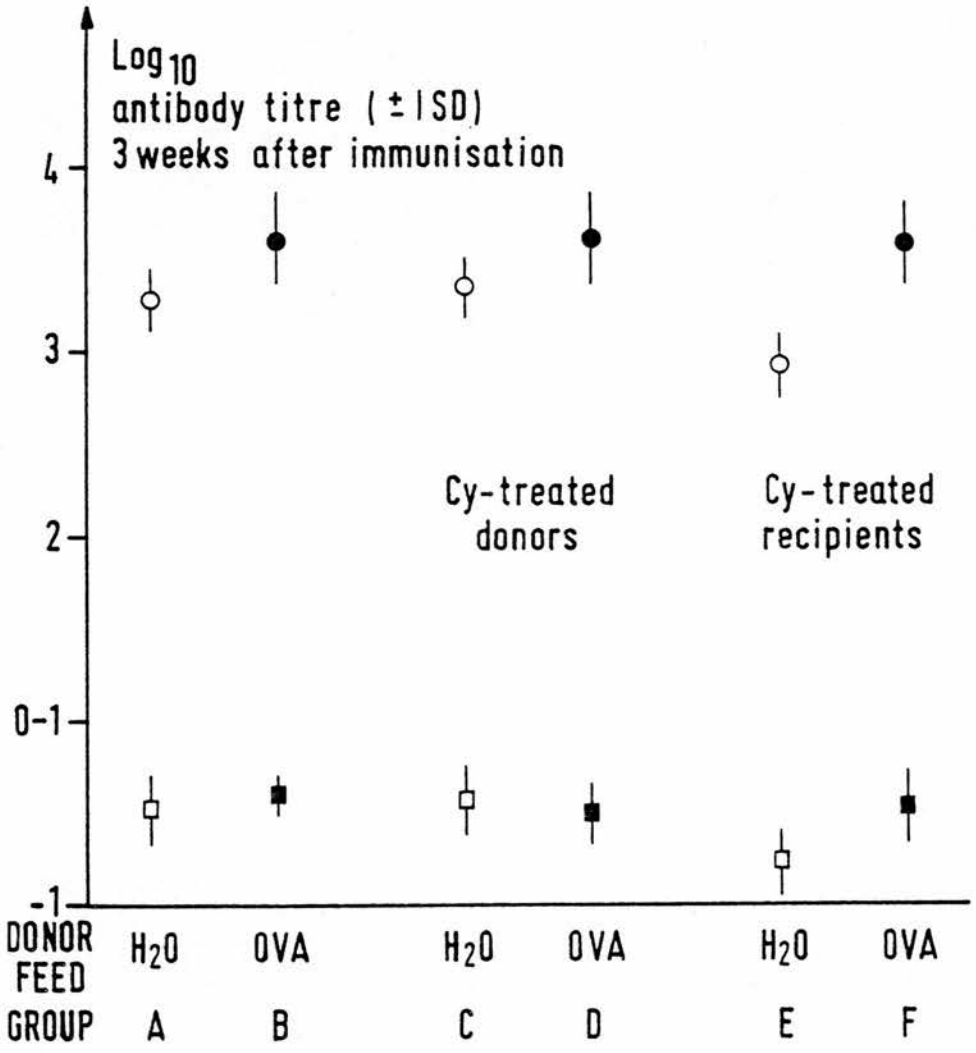
Analysis of the results in recipients of serum from water fed donors (Group A) and ovalbumin fed donors (Group B) showed that serum transfer had no effect on systemic humoral immune responses, the two groups having identical antibody levels, both total and mercaptoethanol sensitive, three weeks after immunisation. Results for a typical experiment are illustrated in Figure 5.3, Groups A and B. In contrast, the mice receiving serum from ovalbumin fed donors showed significant suppression of CMI responses (Figure 5.4, Group B) with 67% suppression ($p<0.02$) when compared with mice receiving serum from water fed donors. This experiment was performed three times with identical results.

3. Effects of cyclophosphamide pretreatment of donors on immune responses of recipients

The serum from cyclophosphamide pretreated and ovalbumin fed donors induced a similar degree of tolerance for CMI in the recipients (Figure 5.4, Group D, 87% suppression when compared with Group C, $p<0.01$). Serum antibody levels were identical to Groups A and B (Figure 5.3).

4. Effects of cyclophosphamide on the immune responses of recipients

Recipient Groups E and F received cyclophosphamide two days before serum transfer. As shown in Figure 5.4, this pretreatment abrogated the immunological tolerance for CMI which was induced when similar serum was transferred to saline pretreated recipients (Group F compared with Group B). No significant effects on serum antibody responses were

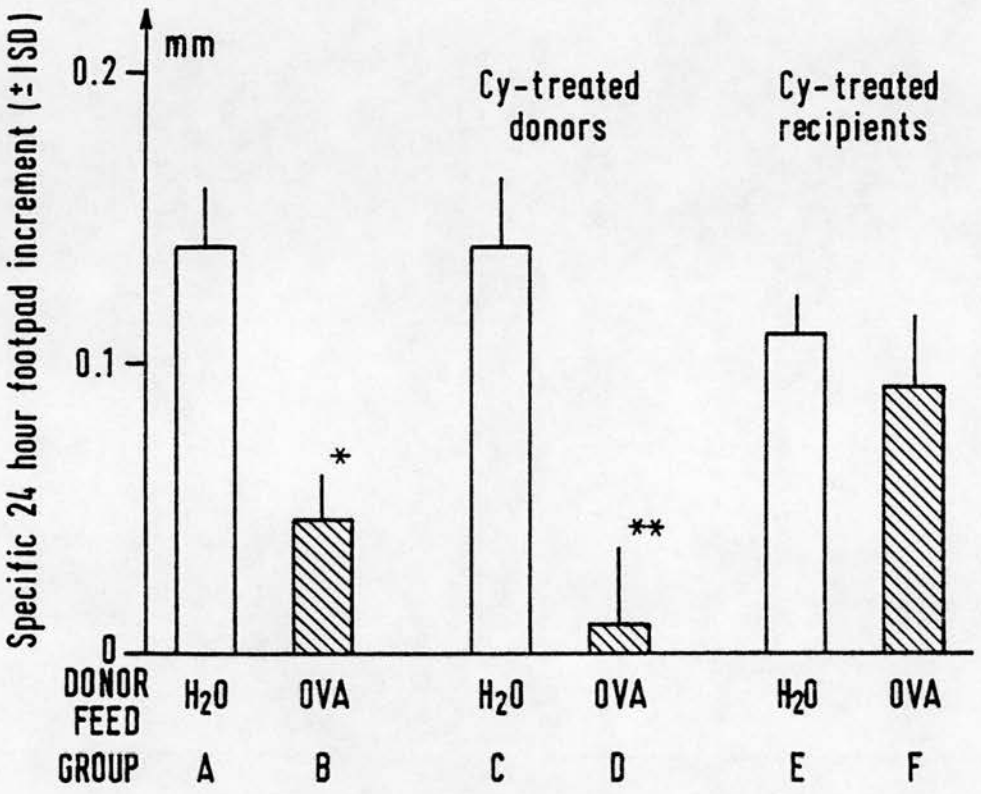


5.3 **Effects of serum transfer and cyclophosphamide pretreatment of donors and recipients on serum antibody responses (Groups A to F)**

Serum antibody titres were measured three weeks after immunisation ($\bar{x} \pm SD$).

Open circles: total IgG titres of water fed controls and in ovalbumin fed mice (black circles).

Open squares: 2-mercaptoethanol-sensitive antibody titres (IgM) in controls and ovalbumin fed mice (black squares).



5.4 **Effects of serum transfer and cyclophosphamide pretreatment of donors and recipients on delayed type hypersensitivity responses in recipients (Groups A to F)**

Specific footpad increments three weeks after immunisation (mean + SD). Transfer of serum from ovalbumin fed donors suppressed the response (group B, $p<0.05$) and cyclophosphamide treatment of donors had no effect on this suppression (group D, $p<0.01$). Cyclophosphamide treatment of the recipients abrogated the suppression induced by transfer of serum (group F).

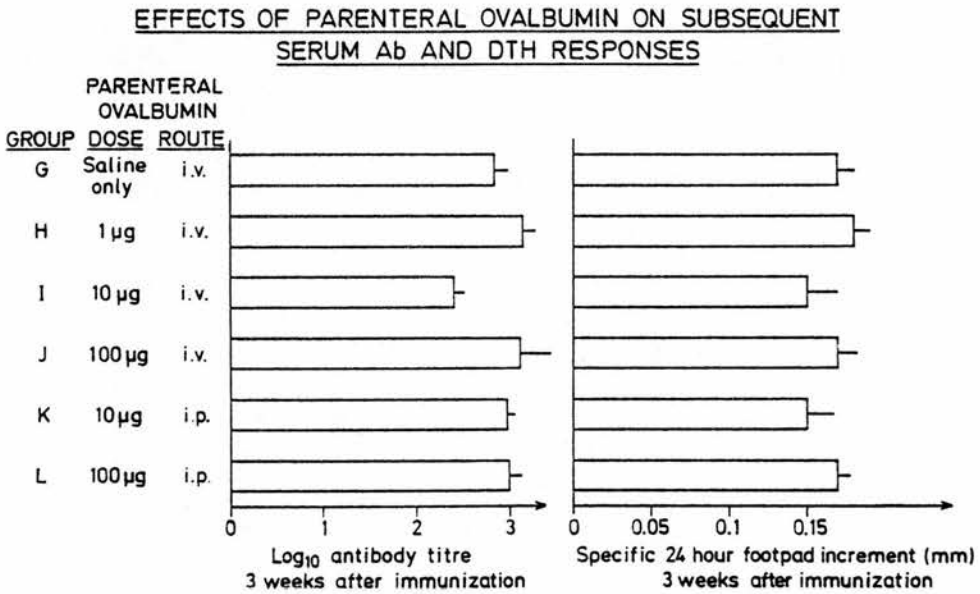
found. The titres of antibody in Group F, although consistently higher than in Group E in three separate experiments, never reached statistical significance.

5. Effects of parenteral ovalbumin on subsequent immune responses of recipients

Unpublished experiments with radioimmunoassay (Hanson, personal communication 1982) and enzyme linked immunoassay techniques (see Chapter 3, Technical developments) indicated that the amount of ovalbumin present in serum after feeding was very variable but likely to be in the range 0.001-1% of the ingested dose/ml mouse serum. Doses of one, 10 and 100 µg native ovalbumin were administered intravenously or intraperitoneally into recipient mice which were then immunised parenterally with ovalbumin in complete Freund's adjuvant and then tested for systemic immunity in the same way as serum recipients (Table 5.2, Groups H to L). As illustrated in Figure 5.5, native ovalbumin used in the doses stated above had no significant effects on the humoral or CMI responses after subsequent parenteral immunisation with ovalbumin and adjuvant.

II. Comments

Oral tolerance is abrogated by cyclophosphamide pretreatment, and the mechanism of abrogation could be either via elimination of T-suppressor cells or via damage to the gut epithelium. A serum transfer protocol has been used to examine roles of the gut in processing fed antigen and to investigate the site of action of cyclophosphamide in relation to oral tolerance for ovalbumin. Serum collected after feeding was transferred into syngeneic recipients. Delayed type



5.5 Effects of parenteral ovalbumin injections on subsequent systemic immune responses

Systemic immunity was measured after injection (i.p. or i.v.) of one, 10 and 100 µg ovalbumin followed by systemic immunisation (groups G to L, Figure 5.2). Native ovalbumin injected in the doses stated above had no effect on humoral or CMI responses.

hypersensitivity responses to ovalbumin in recipient mice were significantly suppressed whereas humoral antibody responses were not affected by serum transfer. Cyclophosphamide pretreatment of recipients (but not of donors) abrogated the immunological tolerance for CMI. Intravenous and intraperitoneal administration of ovalbumin in doses from one to 100 µg did not induce immunological hyporesponsiveness. These results show that, after ovalbumin feeding, humoral and cell mediated limbs of the immune response are likely to be controlled by different regulatory systems. Absorption across the gut mucosa leads to generation of tolerogenic moieties. These tolerogens are stable upon serum transfer and induce hyporesponsiveness for CMI in recipients. This is probably achieved by cyclophosphamide sensitive T-suppressor cells since the effect is completely abrogated by cyclophosphamide treatment of recipients prior to transfer.

B. TRANSFER OF TOLERANCE TO OVALBUMIN BY SERUM OF IRRADIATED DONORS

The interrelationship between ionizing radiation and various aspects of the immune response has been extensively investigated (Taliaferro, Taliaferro & Jaroslow 1964, Anderson & Warner 1975). Small lymphocytes are characterised by their sensitivity to irradiation (Taliaferro et al 1964), small B-cells being more susceptable to damage than small T-cells (Anderson, Sprent & Miller 1974) with cell deaths occuring in the interphase of the cell proliferation cycle. Activated T-cells and T-cells with helper activity are considered radioresistant when irradiated and tested in vivo (Hamaoka, Katz & Benacerraf 1972).

Thus the radiosensitivity of a specific lymphocyte function probably reflects the extent of cell proliferation which must precede the expression of such function (Sprent, Anderson & Miller 1974).

Radiation effects on the intestinal epithelium, which have been reviewed extensively by Quastler and colleagues (1956,1962), are a function of cell population kinetics in a cell renewal system (reviewed by Leblond 1981). Irradiation with 1000 rad or more leads to depletion of the intestinal epithelium mainly by loss of mature cells. Damage after irradiation in the proliferative compartment of the crypt section is seen after 30-60 minutes, the extrusion of cells from the tip of the villus seems to be blocked for the first 12 hours after irradiation. Mucosal damage and epithelial renewal in lethally irradiated rodents lead to villus shortening and crypt lenghthening both of which are seen after a few days. Full histological recovery of the epithelium is achieved after 5-7 days in non-reconstituted rodents. Functional recovery of the epithelium may require up to 17 days (Detrick, Latta, Upham & McCandless 1963).

In the following experiments I shall use the model of intestinal radiation damage as a tool to modulate the immunoresponsiveness of serum donors after ovalbumin feed in a similar adoptive serum transfer system as used earlier (Figure 5.1). Using this experimental design I should gain more insight into the importance of mucosal integrity on the effects of antigen uptake and processing on the subsequent ability to transfer tolerance for CMI. These objectives were pursued quantitively by measuring the circulating amounts of ovalbumin 60 minutes after feeding and qualitatively by a serum transfer protocol measuring systemic immune responses.

I. Experiments and results

The experimental protocol was similar to the one used in the cyclophosphamide study (Figure 5.1) and a standard serum transfer protocol (Table 5.2, group A-D) was incorporated as internal control. All studies were conducted concomitantly.

1. Irradiation

Male and female animals received 1000 rad from a 250 Mega volt orthovoltage X-ray source (Siemens Orthovolt). Mice were housed 6-8 in a perspex cage and were irradiated at a rate of 38 rads/min (the irradiation was carried out in collaboration with Mrs Barbara Clarke, Department of Radiation Oncology, Western General Hospital, Edinburgh).

(a) General health and care of animals after irradiation

Mice were housed in groups of 5-6 in clean cages filled with autoclaved shavings. To prevent weight losses due to dehydration, a bowl with wet solid food was placed into each cage. No other precautions were taken and only two out of 200 mice died within five days after irradiation.

Two days after irradiation: animals were docile, the coat remained shiny and although fecal pellets were softer there were no overt signs of diarrhoea.

Five days after irradiation: animals were docile or lethargic, dehydrated with a ruffled coat and showed signs of overt diarrhoea.

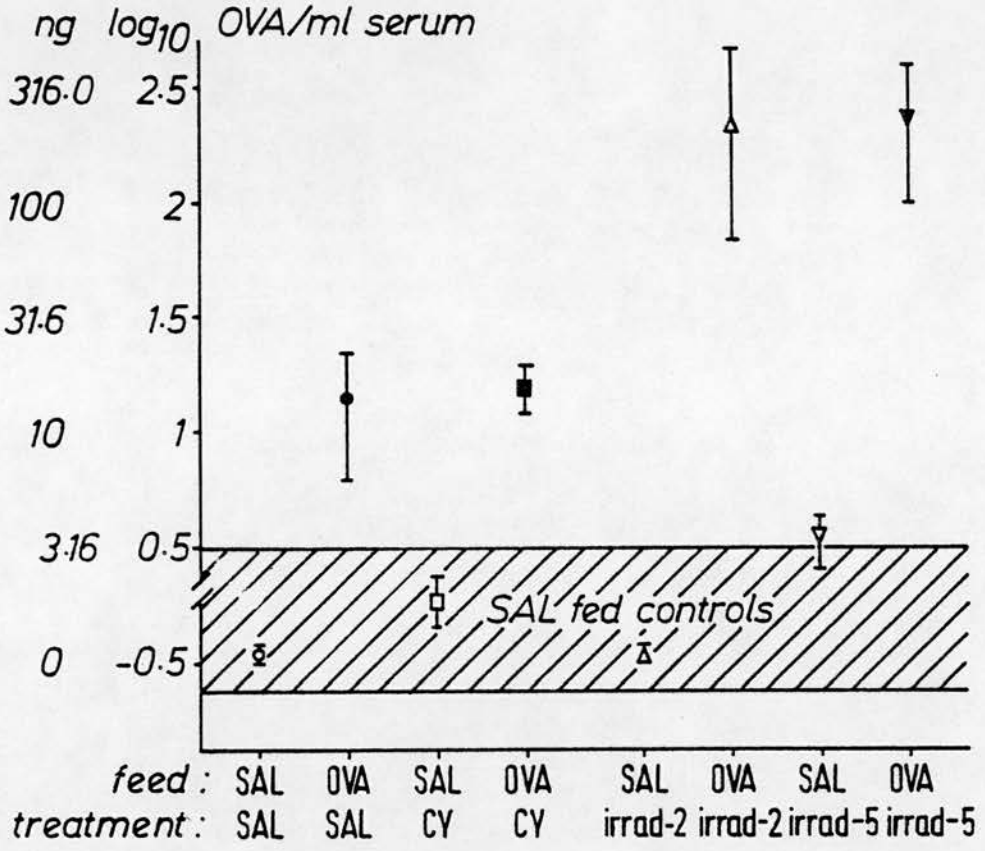
Weights: mice irradiated two days before showed a slight reduction in their body weight (26.8 ± 3.4 g vs. 30.7 ± 3.8 g, $p<0.1$). There was a significant drop of weights in animals irradiated five days earlier ($24.1 \pm .96$ g $p<0.005$).

(b) Uptake of ovalbumin after irradiation and after cyclophosphamide treatment

Ten mice out of each experimental group were fed saline and ovalbumin and their serum was individually assayed for circulating immunoreactive ovalbumin 60 minutes after feeding by using a direct enzyme immunoassay as described (Chapter 3). The results show that cyclophosphamide pretreatment did not alter the amount of circulating antigen compared to saline injected controls (Figure 5.6); 63.7 $\pm$ 50.2 ng/ml vs. 15.4 $\pm$ 3.2 ng/ml serum, pNS, $\bar{x} \pm$ SEM). On the other hand it is evident that irradiation before feeding ovalbumin increases the amount of antigen detectable in the serum two days after irradiation: 228 $\pm$ 162 ng/ml, 5 days after irradiation: 235 $\pm$ 140 ng/ml, $\bar{x} \pm$ SEM) compared to non-irradiated ovalbumin fed mice (63.7 $\pm$ 50.2 ng/ml, $\bar{x} \pm$ SEM, see Figure 5.6).

(c) Comment

It is obvious that intestinal damage was induced two and five days after irradiation. Mucosal injury increased the amount of circulating ovalbumin. The detection of ovalbumin after feeding has been very variable and a similar degree of variability in the serum after feeding has been observed and reported by various other authors (Warshaw et al 1974, Swarbrick 1979, Mowat 1981). Although functional changes in intestinal physiology after irradiation have been described (Detrick et al 1963), it remains to be established whether the increased circulating amounts of a protein found in irradiated mice are due to an enhanced uptake or to a delayed clearance from the circulation caused, for example, by impaired lymph drainage or impaired renal clearance.



5.6 **Circulating amounts of ovalbumin detected in serum after administration of a standard dose (1 mg/g) to normal, irradiated or cyclophosphamide treated mice**

Groups of mice which were irradiated two and five days before feeding ovalbumin exhibited higher serum ovalbumin concentrations than saline or cyclophosphamide treated groups. Note that concentrations are given on a logarithmic scale.

II. Assessment of histological changes at the time of feeding

1. Macroscopic examination:

Two days after irradiation:

On dissection the small bowel was filled with fecal fluid and showed reddening and stiffening of the intestinal walls. The most striking feature was a major reduction in Peyer's patches which were reduced to pin point size. Atrophy of other lymphoid organs was moderate.

Five days after irradiation:

The small bowel of the dehydrated animals was fluid filled, looking very brittle, the intestinal walls were thickened and were torn easily at the attempt of taking specimens for histology. Peyer's patches were no longer visible and lymphoid atrophy was severe.

2. Histological examination:

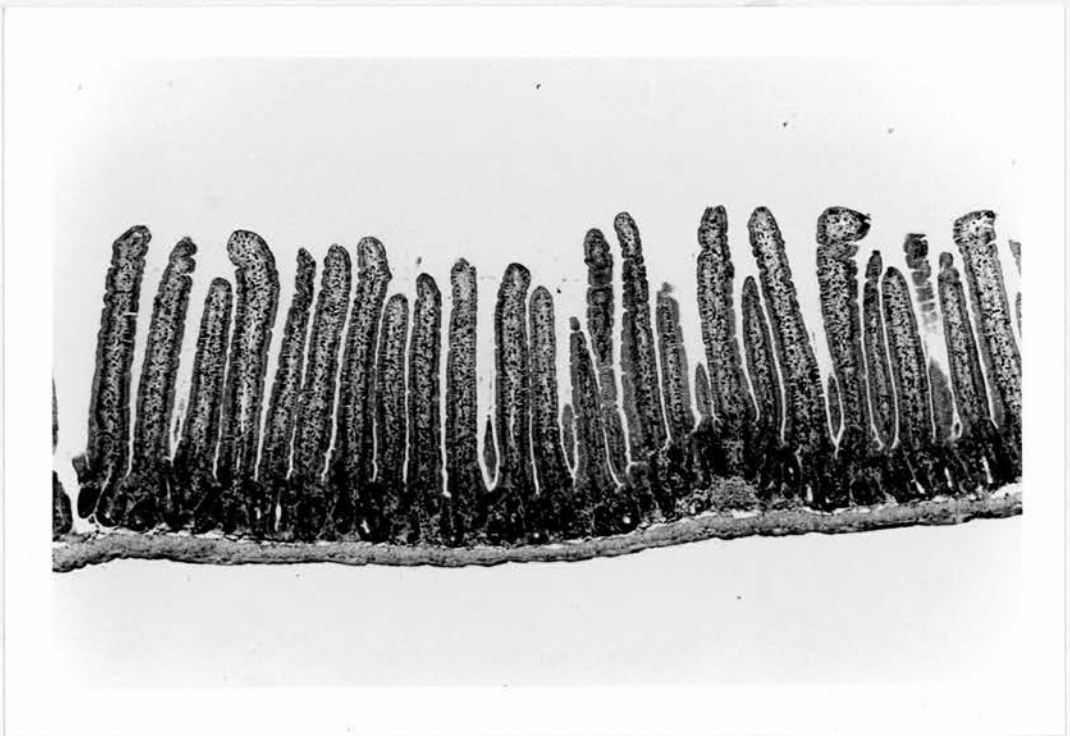
Two days after irradiation (Figure 5.7, normal histology; 5.8a, radiation damage)

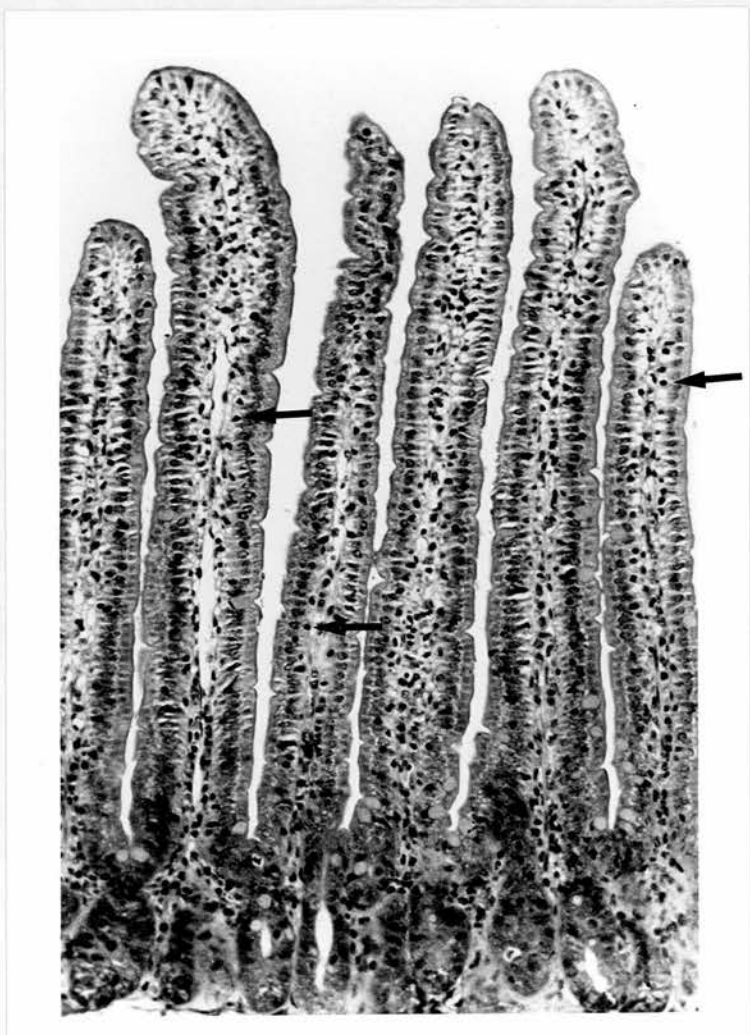
There is universal mucosal damage. The villus pattern is irregular, the villi are shortened, the epithelium is stripping off the basement membrane and some of the epithelial cells are vacuolated.

The crypts are mostly necrotic, shortened and filled with debris. The Paneth cells are disrupted and the cell nuclei are swollen. The Peyer's patch is largely reduced in size and the lymphoid cellular infiltrate has become very sparse.

Five days after irradiation (Figure 5.8b)

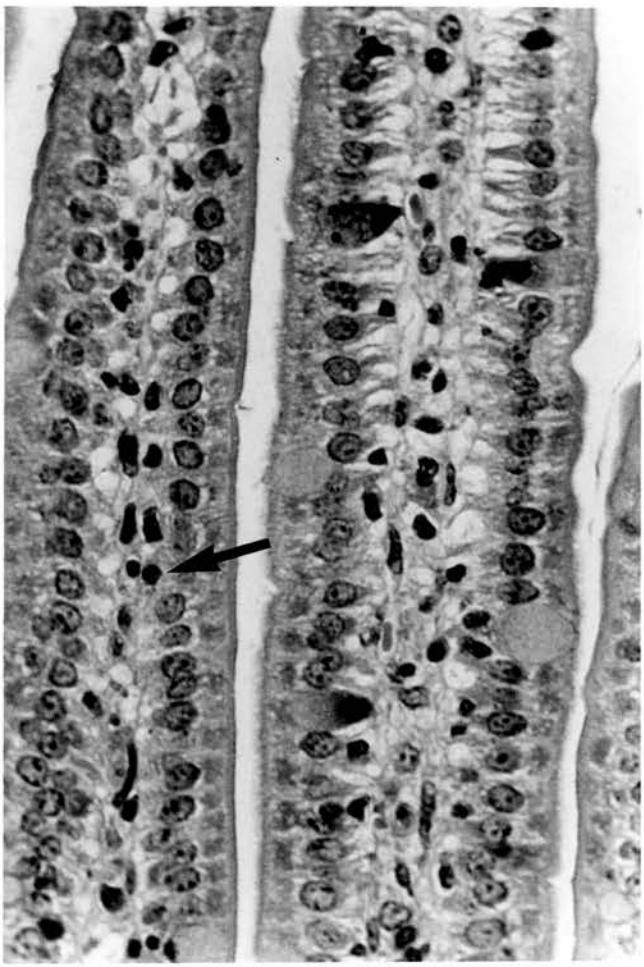
The intestinal damage is patchy with an irregular villus and crypt pattern and a thinned mucosa. There are hyperplastic crypts and areas of villi without crypts. The cellular infiltration of the lamina





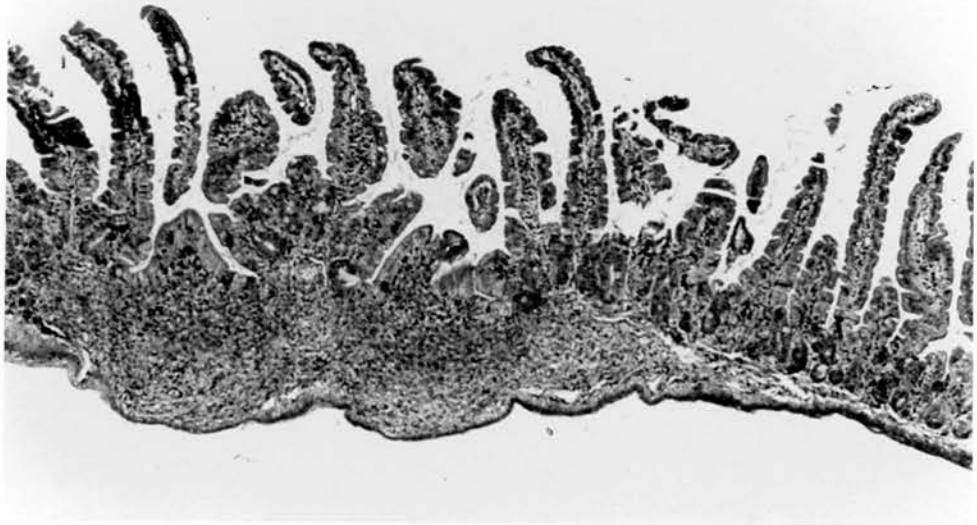
5.7 **Normal jejunal histology (BDF_1 mouse)**

Regular villus pattern with long, finger-shaped villi and normal crypt dimensions (villus to crypt ratio approximately 5:1). Intraepithelial lymphocytes are shown in typical position within the epithelium (arrows - see next page for higher magnification of intraepithelial lymphocytes). Magnification: top x50, bottom x160.



5.7a **Normal jejunal histology (BDF_1 mouse)**

High power view of intraepithelial lymphocytes (magnification x500) in normal numbers and in typical position (arrow; same animal as previous page).



5.8a **Mucosal damage two days after irradiation (1000 rad)**

The normal villus pattern was grossly disturbed, the villi were shortened, epithelial cells were vacuolated and the epithelium was partly stripped off the basement membrane. Crypts were often necrotic, shortened and filled with debris. The Peyer's patches were reduced in size and the lymphoid infiltrate had become very sparse (magnification: top x50, bottom x160).

5.8b **Mucosal damage five days after irradiation (1000 rad)**

The intestinal damage was patchy. The villus and crypt patterns were irregular, the mucosa was thinned and crypt cells were necrotic. The crypts were elongated. Epithelial cells exhibited signs of sub-nuclear vacuolation. The Peyer's patches were further reduced in size and had lost their germinal centres (magnification: top x50, bottom x160).

propria is reduced and focal crypt cell necrosis is present as demonstrated after two days. Again, there is no polymorph infiltration. Many epithelial cells exhibit signs of subnuclear vacuolation. Peyer's patches are grossly reduced in size, have lost their germinal centres, their lymphoid infiltration and consist mainly of reticular tissue.

3. Intraepithelial lymphocyte counts

Irradiated animals showed a progressive drop in intraepithelial lymphocytes from 11.3 $\pm$ 0.9 in controls to 7.0 $\pm$ 6.8 ($p<0.01$) two days and 4.4 $\pm$ 0.91, ($p<0.01$) five days after irradiation (Figure 5.9).

4. Mucosal morphology

Mice irradiated two days before sacrifice showed a decreased villus length (435 $\pm$ 39 µm vs. 576 $\pm$ 41 µm in controls) and crypt length (94.2 $\pm$ 8.0 µm vs. 156.8 $\pm$ 7.1 µm in controls). The villus length had recovered five days after irradiation (536 $\pm$ 56 µm) and hyperplastic crypts showed a significant increase in crypt lengths (323.2 $\pm$ 28.2 µm, $\bar{x} \pm$ SD, $p<0.001$) (Figure 5.9).

III. Effects of serum transfer from ovalbumin fed irradiated donors on systemic immune responses in untreated recipients

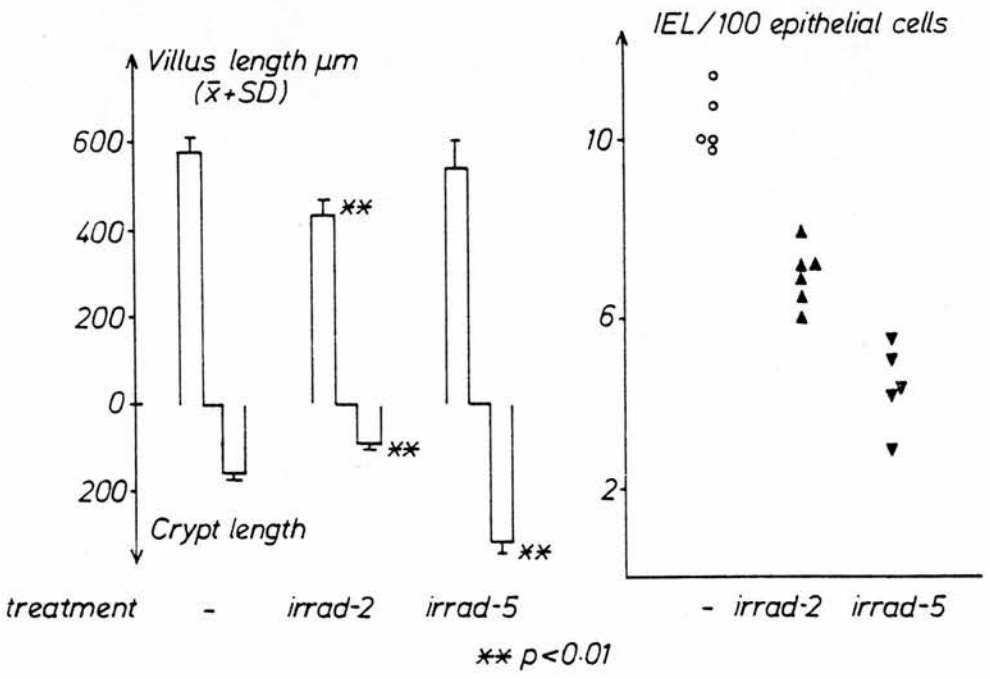
1. Experimental protocol and results

By analogy with the general serum transfer protocol (Figure 5.1), serum donors have either been irradiated (1000 rad) or left untreated. Recipients were not treated except for one internal control group which had been pretreated with cyclosphamide to reverse the tolerogenic effects for CMI after serum transfer, as demonstrated earlier (Figure 5.4).

(a) Humoral antibodies

As in previous experiments, antibody levels are not affected by

Effects of irradiation (1000R) on mucosal morphology



5.9 Effects of whole body irradiation (1000 rad) on mucosal morphology

Two days after irradiation villus and crypt lengths were significantly reduced compared to untreated controls ($p<0.01$). Five days after irradiation crypt length was significantly increased ($p<0.01$), whereas villus length was restored to normal. Intraepithelial lymphocyte numbers showed a progressive decline after irradiation.

transfer of serum from ovalbumin fed donor mice and are shown in Figure 5.10a.

(b) Cell mediated immune responses

Untreated recipients exhibited a marked suppression of their CMI responses (0.030 $\pm$ 0.028 mm vs. 0.105 $\pm$ 0.017 mm, $p<0.001$). Suppression was abolished by cyclophosphamide treatment of recipients. Delayed type hypersensitivity responses in the group of animals which received serum from donors fed ovalbumin were slightly enhanced (0.096 $\pm$ 0.017 mm vs. 0.12 $\pm$ 0.015 mm, $\bar{x} \pm$ SEM, $p<0.05$) (Figure 5.10b). Irradiation, either two or five days before transfer of serum abolished suppression of CMI, which is in sharp contrast to the effects seen after serum transfer from non-irradiated donors.

Similar to the effects seen with cyclophosphamide, transfer of serum of both irradiated donor groups evoked slight priming effects in the recipients:

Two days after irradiation: 0.088 $\pm$ 0.02 mm vs. 0.11 $\pm$ 0.02 mm

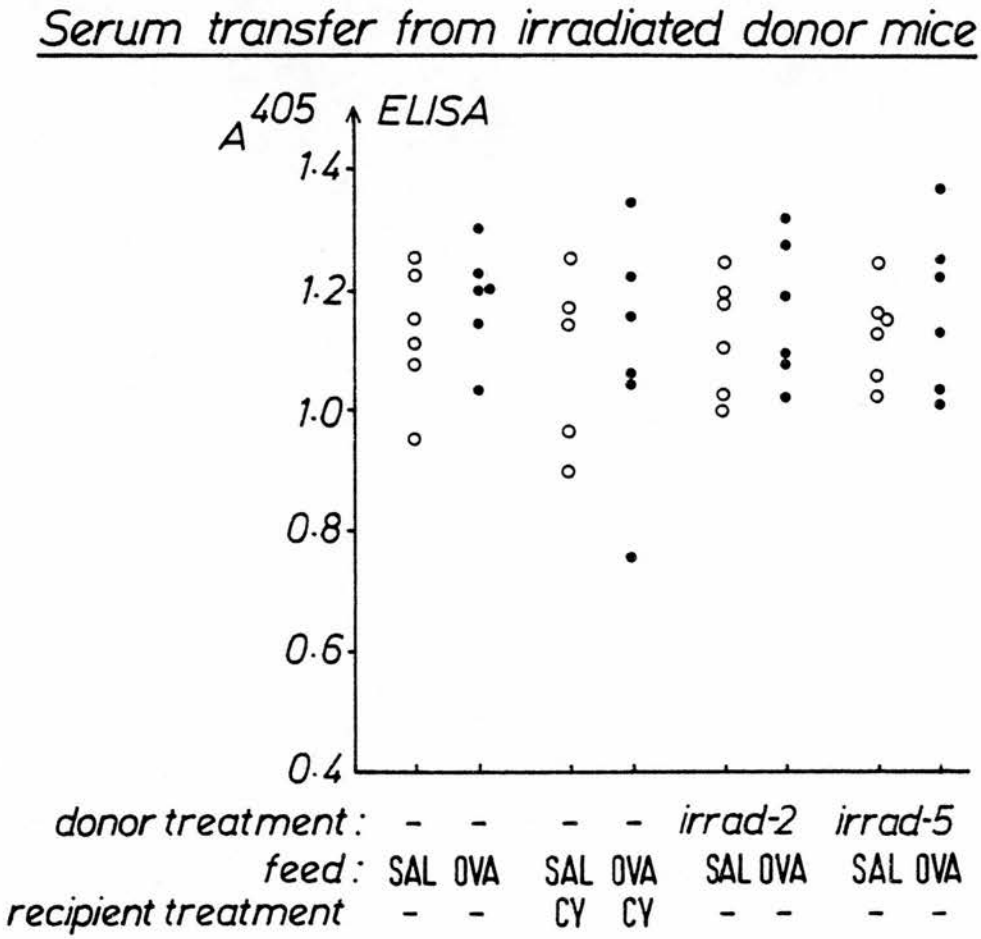
Five days after irradiation: 0.13 $\pm$ 0.008 mm vs. 0.14 $\pm$ 0.03 mm,

$\bar{x} \pm$ SEM, pNS) (Figure 5.10b).

IV. Comment

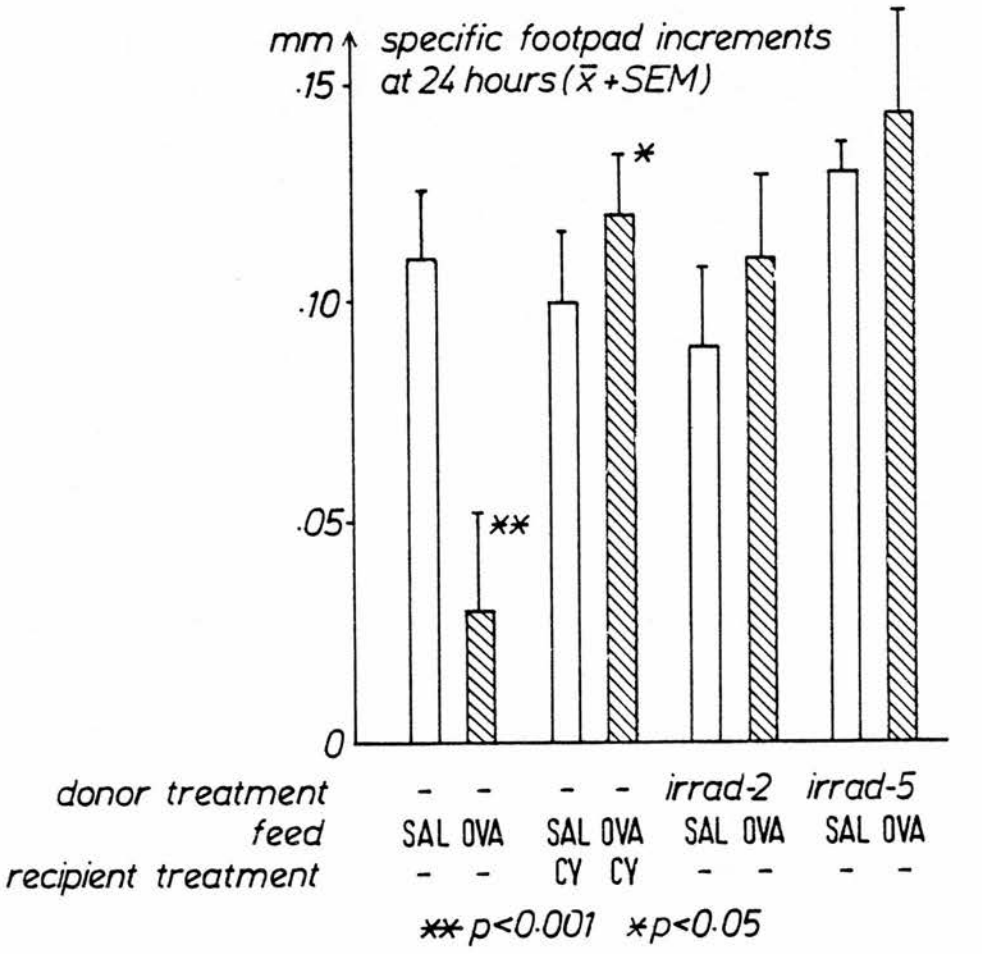
Whole body irradiation caused severe intestinal mucosal damage, led to lymphoid atrophy with progressive decline of Peyer's patch size and of intraepithelial lymphocyte numbers. Although the animals were severely compromised both immunologically and intestinally, the mortality was only 2/200 which is an index of the clean animal housing conditions and good animal care.

The increased amounts of circulating ovalbumin found in the serum



5.10a **Systemic immune responses in BDF_1 recipients after serum transfer from irradiated donor mice**

Humoral antibody responses.



5.10b **Systemic immune responses in BDF$_1$ recipients after serum transfer from irradiated donor mice**

Delayed type hypersensivity responses: CMI responses were suppressed by transfer of serum from ovalbumin fed donors ($p<0.001$). The suppression was abrogated by cyclophosphamide treatment of recipients (see also Figure 5.4). Irradiation two and five days before serum transfer (donors) abrogated the transferable tolerance.

of irradiated mice could have been evidence for either increased uptake or a decreased clearance, for example, due to obstruction of lymphatics or to renal impairment. The exact mechanism, however, remains to be established. All of the above mentioned features are likely to have interfered with the transfer of tolerance for CMI by serum of irradiated and ovalbumin fed donors. The next step in elucidating this phenonomen would be to investigate the effects of immediate reconstitution after irradiation, and to examine qualitatively the serum of irradiated and ovalbumin fed mice and to find out whether the hypothetical balance between immunogenic and tolerogenic fragments has been altered.

Chapter 6

EFFECTS OF AGE ON INDUCTION OF ORAL TOLERANCE

Patients with food allergic diseases exhibit active systemic immunity to food antigens - implying that instead of oral tolerance, priming or sensitisation has resulted from previous enteral encounter with the antigen. The induction of a comparable abnormal immune responses in animals would provide a model for investigation of this group of important diseases, and since food allergic disease in humans is a feature of infancy, there is special interest in defining the mechanisms which regulate immune responses to fed antigens in neonates.

Immunological tolerance is very readily induced in neonatal animals when antigen is given by routes other than via the gut. This has been demonstrated with many different forms of antigen, including allogeneic cells (Billingham et al 1953, Medawar 1961), skin sensitising agents (Ptak & Skowron-Cendrzak 1977) and aggregated human gamma globulin (Etlinger & Chiller 1979). In view of this relative ease of tolerance induction in the neonatal period it might be expected that oral tolerance would normally be even more profound and complete in neonates than in adults. However, in the only report of relevant experiments, in which the antigen ovalbumin was fed to neonatal mice, Hanson found priming for subsequent humoral responses, and not oral tolerance (Hanson 1981). The effects of neonatal feeding on subsequent CMI responses have not been described. I have therefore undertaken a series of experiments to examine how the age of an animal influences specific systemic immune responses to a fed antigen - resulting in either oral tolerance or oral sensitisation (priming).

A. EXPERIMENTAL DESIGN AND RESULTS

Experiments were designed to investigate the following immunological consequences of a feed of either antigen or saline in the neonatal period:

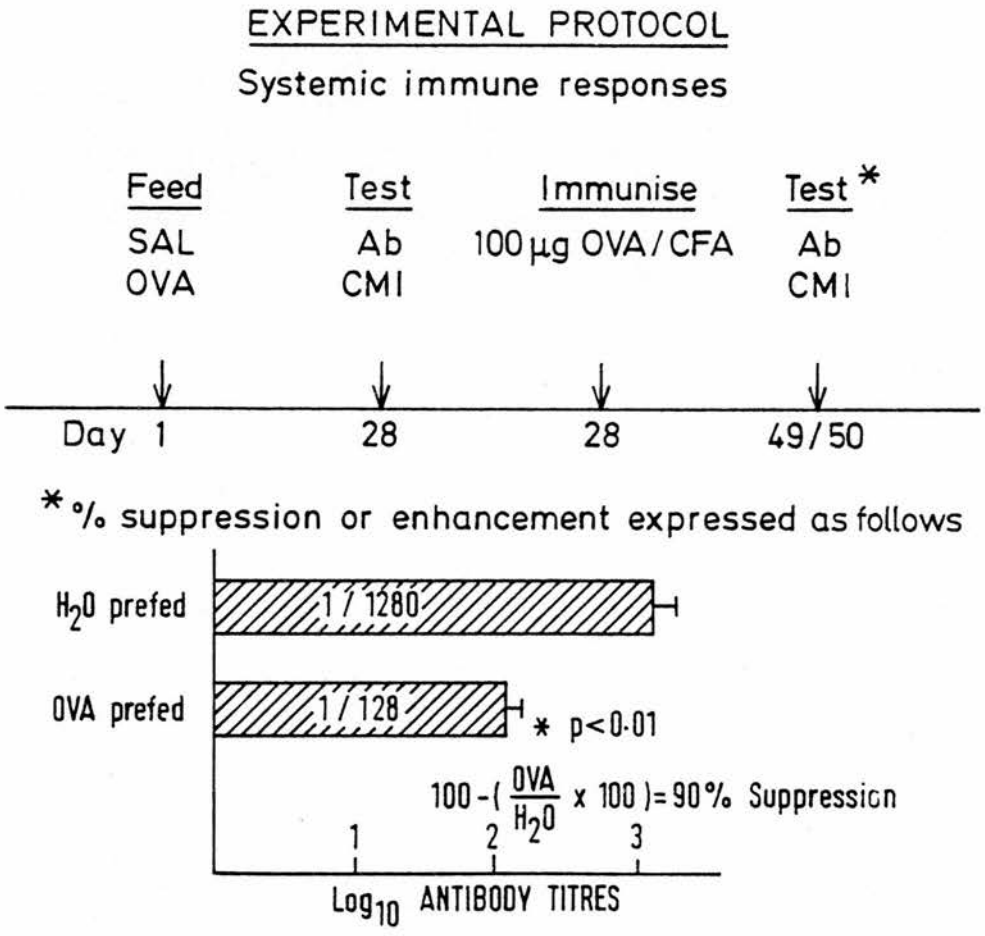
1. Generation of systemic immunity by feeding.
2. Induction of priming (ie. enhancement of the immune response to a parenterally administered antigen in animals without antibody responses or CMI before parenteral immunisation).
3. Suppression (tolerance) of the immune response when antigen fed animals are compared to controls. Suppression was calculated by using the following formula:

$$\%\text{suppression} = 100 - \left[\frac{\text{response of antigen fed mice}}{\text{response of saline fed mice}} \times 100\right]$$

Animals were fed ovalbumin or saline by intragastric intubation or by intraamniotic injection 24-36 hours before birth (see Materials and Methods). Four weeks later, serum was tested for antibodies, and skin tests were performed to detect CMI responses induced by this initial treatment (these tests were uniformly negative). All animals were then immunised with ovalbumin in complete Freunds's adjuvant, usually aged 28 days and systemic immunity tested as before (Figure 6.1).

I. Age of the pups

For defining the age of the pups, the breeding pairs were checked twice per day (9 am, 5 pm) and litters delivered before 9 am were assigned a birth date corresponding to the preceding day. Animals which were treated at day one were placed in experiments on the morning they were discovered and were thus aged between 16-24 hours.



6.1 **Experimental protocol for the investigation of age related effects on induction of oral tolerance**

II. **Induction of systemic immunity by feeding**

Feeding of antigen did not induce systemic immunity when this was tested in three separate experiments.

III. **Age of animals at the time of immunisation**

Due to the experimental protocol and necessary adult control animals, the age of the animals at the time of immunisation ranged from four weeks (neonatal feeding experiments) to 16 weeks when the long term effects of a neonatal feed were investigated. Adult animals (4 weeks of age) were immunised seven days after the initial feed. Nevertheless, a comparable state of systemic immunisation or tolerance was obtained in all appropriate controls within the age range described.

B. INFLUENCE OF AGE AT FIRST FEED ON TOLERANCE INDUCTION

These experiments were performed to document the time course of tolerance induction, to test the observed phenomenon in several strains, and to confirm a previous report (Hanson 1981) that there is transient reduction in oral tolerance for antibody when mice are fed antigen around the time of weaning.

I. **Intragastric ovalbumin administration to neonatal, immature and adult mice**

Mice were fed ovalbumin in a weight related dose (1 mg/g body weight) by intragastric intubation at the ages of one, three, seven, 14 and 42 days. Animals fed ovalbumin between one and seven days after

birth did not develop oral tolerance. Indeed, mice fed one day after birth exhibited, repeatedly and consistently, signs of priming both for antibody responses and CMI (Figures 6.2 and 6.3). Extrapolation from these data suggest that the crossover to significant tolerance induction occurs around the tenth day after birth.

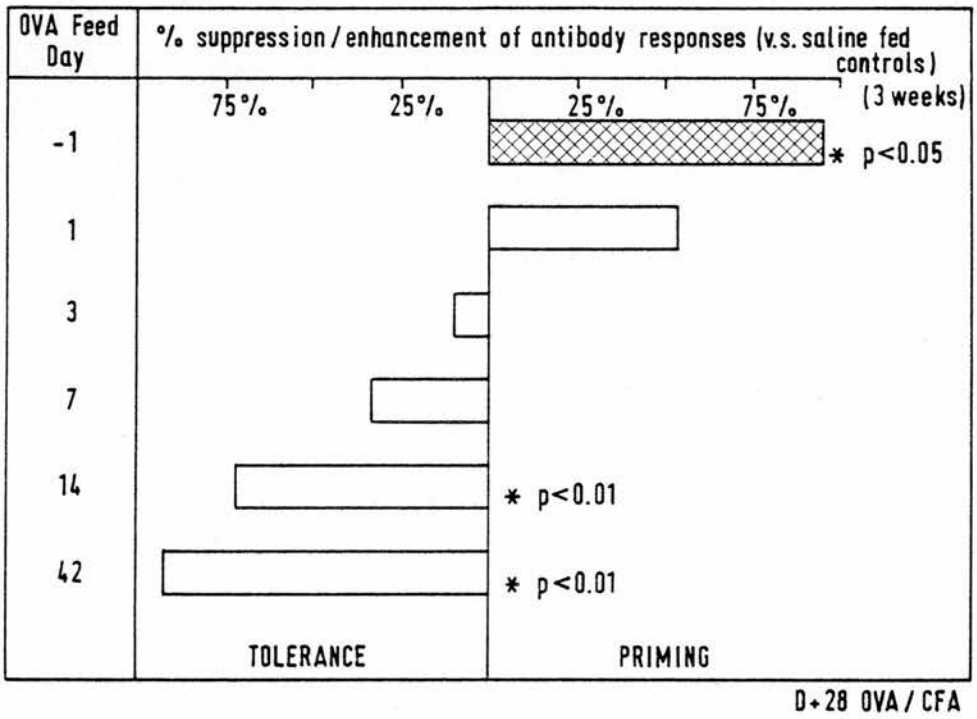
II. Effects of ovalbumin feeds at day one on systemic immune responses in different inbred mouse strains

In order to ensure that the observed priming effect by feeding antigen on the first day of life was not specific for only one strain, two other strains of mice (CBA and BALB/c) were also tested. Before these experiments were started, it had been shown that adult mice of each of these strains were tolerised to a similar degree by a single weight related dose of ovalbumin (suppression of humoral responses range from 69-88% and of CMI 74-93%; see Chapter 4). Feeding of ovalbumin on the first day of life led to qualitatively similar priming effects for both systemic antibody and CMI responses in repeated experiments (Table 6.4).

III. Effects of weaning on the induction of tolerance to ovalbumin

By the time mice were aged 14 days, the magnitude of immunological suppression produced by a feed of ovalbumin was as complete as in adult animals. However this pattern was disturbed when animals were fed during the weaning period. In order to separate the effects of age and those of weaning, animals were fed ovalbumin or saline on the day of weaning, and three and seven days on either side of this day. In some groups weaning was delayed for three days so that animals of identical

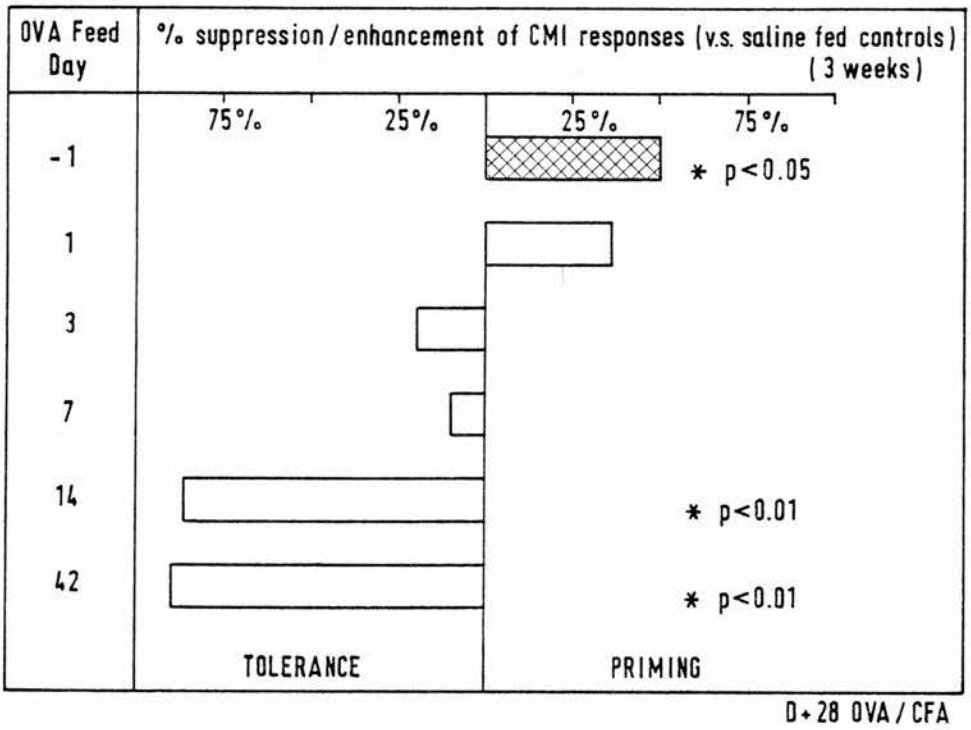
Effects of OVA feeds at various ages on subsequent immune responses of BDF_1 mice



6.2 **Effects of ovalbumin feeds at various ages on the subsequent immune responses of BDF_1 mice**

% suppression or enhancement of humoral antibody responses three weeks after immunisation compared to age matched and sham treated controls. Prenatal (intraamniotic) antigen exposure enhanced the subsequent immune response ($p<0.05$).

Effects of OVA feeds at various ages on subsequent immune responses of BDF_1 mice



6.3 **Effects of ovalbumin feeds at various ages on the subsequent immune responses of BDF_1 mice**

% suppression or enhancement of CMI responses three weeks after immunisation compared to age matched and sham treated controls. Prenatal (intraamniotic) antigen exposure enhanced the subsequent immune response ($p<0.05$).

Table 6.4

Enhancement of immune responses in different mouse strains after feeding ovalbumin on the first day of life

Strain	Feed	Age (days)	% Enhancement [a] of Antibody responses	% Enhancement [a] of Cell mediated immunity
CBA	OVA	1	+54,+10	+36,+25
BALB/c	OVA	1	+41	+26
BDF_1	OVA	1	+51,+41*,+16	+24,+31,+4

a) % enhancement of systemic immune responses compared to saline fed littermate controls

All animals were immunised four weeks after birth

* $p<0.01$

age differed only in their weaning date. The results are summarised in Table 6.5. When mice were weaned at age 21 days and given an ovalbumin feed on that day, they showed no subsequent oral tolerance for antibody responses, and less than usual suppression of CMI responses. On the other hand, littermates weaned three days after feeding, or fed ovalbumin three days after weaning, showed significant oral tolerance both for humoral and CMI responses. When there was an interval of seven days between the day of weaning and ovalbumin feed, oral tolerance analogous to that found in adult animals, was observed. It is therefore clear that the transient absence of oral tolerance in these mice aged 21 days was not age related, but the result of weaning.

C. INFLUENCE OF PRENATAL ANTIGEN EXPOSURE

The following experiments were designed to give "oral" and "parenteral" antigen in utero, by injecting ovalbumin into the amniotic sack (feeding) and by using intravenous injection of a pregnant female as a means of exposing the pups, systemically, to antigen. The experiments were carried out to examine whether the consistent priming, observed in animals fed antigen on the first day of life, is increased after prenatal exposure. These experiments would also give useful information on the capacity of a mouse foetus of 19 days gestation to mount antigen specific immune responses after in utero antigen encounter.

I. Effects of prenatal intestinal antigen exposure

The weight of a mouse foetus on the 19th day of gestation is around 1 g. The foetuses were therefore exposed to 1 mg ovalbumin by intra-

Table 6.5

Effects of ovalbumin feeding on tolerance induction in relation to age and time of weaning

Age	Day of feeding in relation to weaning	n	antibody responses		cell mediated immunity	
			% suppression [a]	p<	% suppression [a]	p<
14	-7	8	70	.02	92	.001
21	-3	10	52	.05	97	.001
21	0	7	28	NS	33	.05
24	+3	8	43	.05	75	.001
28	+7	8	82	.01	90	.001

a) % suppression of systemic immune responses after feeding 1 mg/g ovalbumin compared to saline fed littermate controls

All animals were immunised with 100 µg ovalbumin in Freund's complete adjuvant 14 days after feeding

n) Animals per group

amniotic injection in 0.05 ml. Results presented in Figure 6.6 show that by extending the timescale of feeding experiments into uterine life, priming of the offsprings was further increased when compared with the day one fed animals. As the amount of ovalbumin which was in fact taken up by the foetus could not be exactly measured, the results are not completely comparable with the effect of closely controlled postnatal feeds. However, they demonstrate clearly that antigen administration to foetuses through the amniotic fluid does lead to an active priming of the immune response and that this effect extends into adult life.

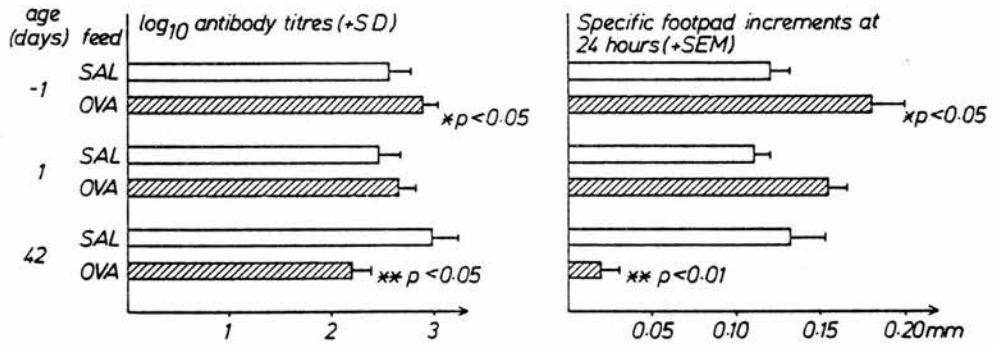
II. Effects of intravenous ovalbumin injections into pregnant females on their pups' susceptibility to tolerance induction in later life

Pregnant females were injected with 8 mg ovalbumin intravenously, 24-36 hours before delivery. The subsequently born pups were fed saline or ovalbumin four weeks after birth. All animals were then immunised and antibody and CMI responses measured as described earlier. The results of these experiments (Table 6.7) show that intravenous ovalbumin injection, in amounts similar to those used for intraamniotic antigen administration, did not enhance immune reactivity in the pups. Tolerance induction, produced by protein feeding at the age of 28 days, was identical in the pups of saline or ovalbumin injected mothers, both for humoral and CMI responses.

III. Specificity of priming by intraamniotic antigen injection

The priming effects described above have been shown to be antigen-

Effects of intra-amniotic OVA application on systemic immunity compared with postnatal controls



6.6 Effects of intraamniotic ovalbumin administration on systemic immunity compared with controls fed postnatally

Mice being exposed to ovalbumin during foetal life by intraamniotic injection exhibited significantly enhanced antibody and CMI responses ($p<0.05$) when tested at four weeks of age. Animals fed ovalbumin at day one of life showed consistent, although not significant, enhancement of systemic immunity. Adult mice developed classical oral tolerance.

Table 6.7

Effects of intraamniotic and maternal intravenous injection of ovalbumin or saline on subsequent immune responses of BDF_1 mice

Injection at age - 1 day	Feed at age 28 days	Specific 24 hours footpad increments (mean ± SEM)	ELISA absorbance at 405 nm (mean ± SEM)	Animals per group
intraamniotic				
saline	saline	0.14 ± 0.01	0.523 ± 0.18	6
saline	ovalbumin	0.03 ± 0.004[a)]	0.317 ± 0.05[b)]	5
intravenous (mother)				
8 mg ovalbumin	saline	0.13 ± 0.01	0.543 ± 0.050	6
8 mg ovalbumin	ovalbumin	0.04 ± 0.01[a)]	0.311 ± 0.0.41[b)]	6

a) $p < 0.001$

b) $p < 0.01$

All animals were immunised with ovalbumin (100µg) in CFA seven days after feeding.

specific since intraamniotic administration of bovine serum albumin did not lead to enhancement of immune responses against ovalbumin.

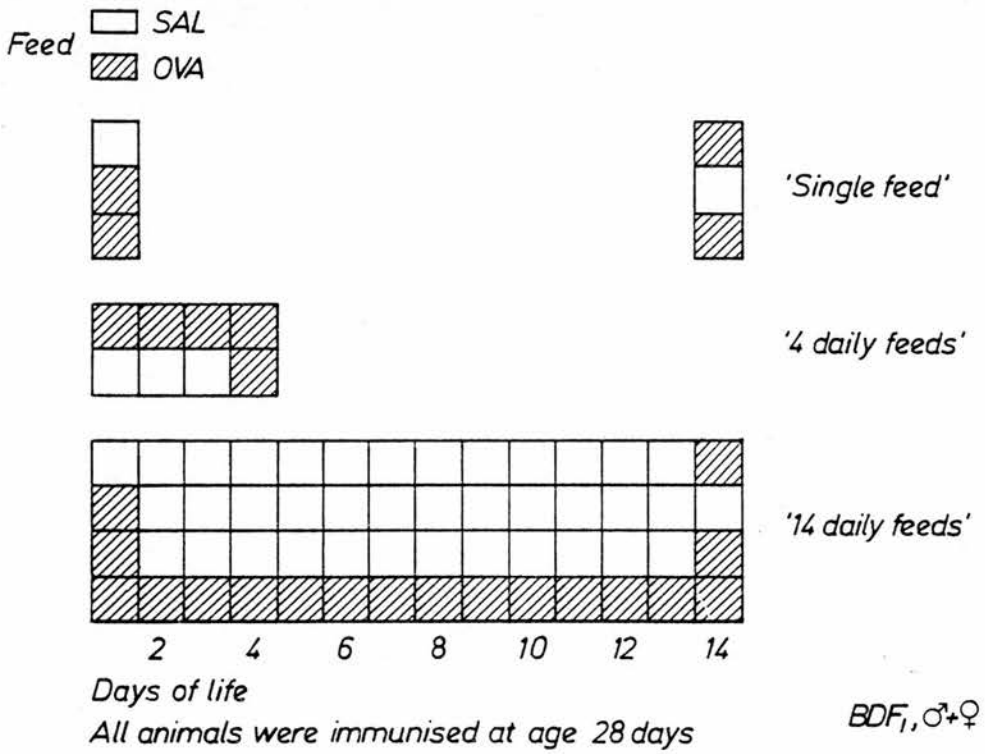
D. ATTEMPTS TO TOLERISE NEONATALLY PRIMED ANIMALS

A series of experiments were performed, in order to determine whether it is possible to modulate subsequent immune responses in animals which had been given a priming dose of ovalbumin in the neonatal period. Further feeds of ovalbumin were given to the animals, either in the first few days of life or in young adult life. Theoretically, this could have led either to an increase in the priming effect or to subsequent recreation of oral tolerance. It was particularly important to examine whether primed animals could be retolerised by one or more feeding regimens which extended into the period of life where young animals were usually tolerisable (by age 14 days).

I. Effects of age, frequency and total amounts of neonatal ovalbumin feed on subsequent immune responses

The different feeding schedules and antigen doses are schematically summarised in Figure 6.8. Animals were fed ovalbumin or saline on the first day of life and four or 14 days thereafter. Two groups of animals were fed daily for four and 14 days. The effect of antigen dose was examined by feeding the total amount which had been given to these groups of animals over four or 14 days, in a single dose on the fourth or 14th day of life. Sham fed control groups were incorporated into these experiments. They were immunised on the same day as the relevant experimental groups. For simplicity, the groups have been identified A to G (see Table 6.9). Group A (one feed, day one),

Age dependant effects of an OVA feed on systemic immunity
EXPERIMENTAL PROTOCOL



6.8 Experimental protocol for testing age-dependant effects of ovalbumin feeding on systemic immunity

Groups of animals were assigned to three different feeding regimens as indicated.

Table 6.9

Effects of feeding frequencies and antigen doses on subsequent systemic immunity

	Experimental groups	Total mg OVA	mg OVA/ g body weight	Age at 1st feed (days)	Feed	Age at last feed (days)	Feed	Feeding x n	Mice n	% Suppression or enhancement (+)[a] Antibody responses	p	CMI responses	p
A	One day 1 feed	2	1	1	OVA	14	SAL	x1	8	+41	<.01	+11	NS
B	Two feeds (day 1,14) low dose control	8	1	1	OVA	14	OVA	x2	11	+ 5	NS	22	NS
C	Four daily feeds (1-4)	8	1	1	OVA	4	OVA	x4	7	96[b]	<.001	18	<.1
D	One feed day 4	8	3	1	SAL	4	OVA	x1	9	33[b]	NS	10	NS
E	14 daily feeds	50	1	1	OVA	14	OVA	x14	11	28	<.02	96	<.01
F	One feed day 14	50	7	1	SAL	14	OVA	x1	8	28	<.02	89	<.01
G	Two feeds (day 1,14)	50	7	1	OVA	14	OVA	x2	11	50	<.01	28	NS

a) Effects on systemic immune responses after ovalbumin feeds compared to saline fed littermate controls

b) Results obtained with passive haemagglutination assay

demonstrates that the priming effect of a single antigen feed is still detectable after two weeks, and Group B (two feeds, day one, day 14) shows that a single refeed does not reverse this effect. However the group of mice which was fed daily for the first four days of life (Group C, four daily feeds) showed suppression of their antibody responses ($p<0.001$) and reduced, although not significantly suppressed CMI responses ($p<0.1$), whereas mice receiving an identical total quantity of ovalbumin in a single dose on the fourth day of life (Group D, one feed day four) showed no suppression of their immune responses - ie. were not tolerant.

In order to investigate the effects of daily feeds in more detail, littermates were fed 1 mg/g body weight for 14 days (Group E, 14 daily feeds) or received the same amount of ovalbumin (50 mg) on one occasion (Group F, one feed day 14). One feed at age 14 days tolerises an animal as expected, for both antibody and CMI responses (Group F). However the same quantity of antigen given in divided doses on day 1 and day 14 of life only suppressed subsequent antibody responses (Group G). When the same quanity of ovalbumin was split into 14 daily doses (Group E), animals were subsequently tolerant. This implies that multiple daily feeds of ovalbumin are more effective than a single feed of the same total amount in suppression of delayed type hypersensitivity responses.

II. Persistence of priming in animals which were fed ovalbumin on the first day of life and refed ovalbumin at various times thereafter

Animals were fed ovalbumin on the first day of life and attempts were made to retolerise these mice by an ovalbumin feed two, four, six,

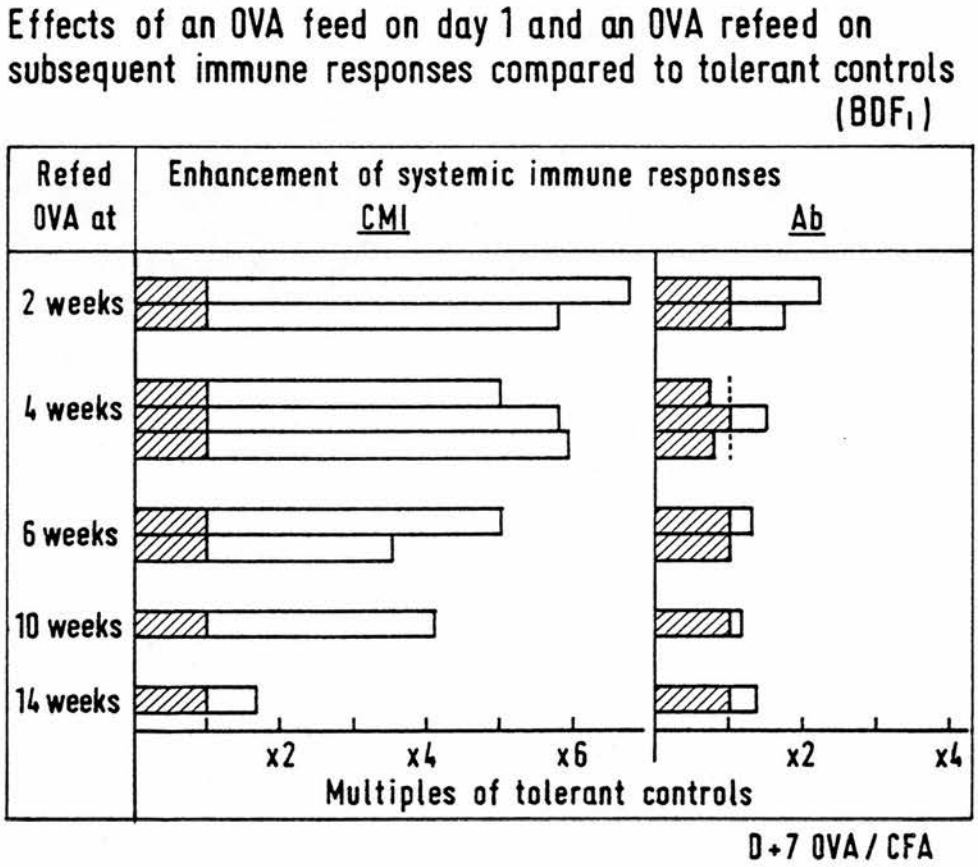
10 and 14 weeks after the initial treatment. The results are illustrated in Figure 6.10. Primed antibody responses were found to be partially suppressable by feeding only two weeks after the initial priming dose and were indistinguishable from those of tolerant animals when retolerisation was performed four weeks after the initial treatment. On the other hand, CMI responses exhibited a striking disparity in that retolerisation was not accomplished before 14 weeks.

III. Persistence of priming in animals which received intraamniotic ovalbumin and were refed at age 28 days

Ovalbumin or saline were injected intraamniotically 24-36 hours before birth and the pups were subsequently fed ovalbumin or saline at age 28 days (Table 6.11). Results of the 'intraamniotic saline' group show that this manipulation had no effect on the capacity of the pups to respond in a normal manner (by oral tolerance) when ovalbumin was fed at age 28 days. On the other hand, animals which had been exposed to ovalbumin by intraamniotic injection demonstrated oral tolerance for serum antibody responses when retolerisation was attempted by feeding antigen four weeks after birth; tolerance for CMI responses, however, was not present in the same animals (Table 6.11). These experiments demonstrate that prenatal antigen exposure had similar effects on subsequent immune responses as did feeding on the first day of life.

E. COMMENT

Substantial priming effects seen after ovalbumin feeds in the neonatal period and the failure to induce tolerance by an important



6.10 **Effects of an ovalbumin feed to one day old mice followed by ovalbumin challenge on their subsequent immune responses compared with tolerant controls**

In slight deviation from the general protocol, animals received ovalbumin or no treatment on the first day of life and were fed ovalbumin at various times thereafter. The stippled line indicates the mean humoral immune response at four weeks. The hatched bars depict the immune responses of tolerant mice and results are given as a multiplication factor of the responses of tolerant animals.

Table 6.11

Effects of intraamniotic ovalbumin injections followed by an ovalbumin feed at 28 days on the subsequent immune responses of BDF_1 mice

Group	Intraamniotic injection at age -1 day	Feed at age 28 days	Specific 24 hour footpad increment (mean ± SD)	Log_{10} antibody titres 3 weeks after immunisation (mean ± SD)	Animals per group
A	saline	saline	0.17 ± 0.03[a)]	3.29 ± 0.22	5
B	saline	ovalbumin	0.03 ± 0.01	2.60 ± 0.20[c)]	5
C	ovalbumin	ovalbumin	0.14 ± 0.03[b)]	2.59 ± 0.19[c)]	5

a) $p<0.001$ compared to Group B

b) $p<0.005$ compared to Group B

c) $p<0.01$ compared to Group A

physiological route of antigen exposure is in striking contrast to the generally recognised pattern - obtained from animal work - that neonatal mice are very readily tolerised if the antigen is administered via non-enteral routes, ie. intravenously, intraperitoneally, intradermally (Billingham et al. 1953, Chiller & Weigle 1971, Etlinger & Chiller 1979).

The crossover from priming or non-tolerance to tolerance is around the 10th day of life and the increasing ease of tolerance induction was briefly interrupted around the time of weaning and was related to the time interval between weaning and feeding and not to the age of the animals. My attempts to retolerise initially primed animals showed that daily feeds of a priming dose for 14 days were capable of retolerising animals, whereas the same dose fed only twice was not. Furthermore, it is obvious as shown in Figure 6.10 that systemic humoral antibody responses are more easily retolerised than systemic CMI responses. These experiments however did not show and were not designed to reveal whether the abrogation of oral tolerance would, on the same hand, induce a local mucosal CMI.

The demonstration of such a connection would be the missing link for using the neonatal feeding experiments as an animal model to investigate the mechanisms of food related mucosal changes which can be induced by antigen feeds in human infants.

Experiments designed to investigate how a mucosal CMI can be induced and investigated will be described in the next chapter.

Chapter 7

INDUCTION OF MUCOSAL CELL MEDIATED IMMUNITY

A. GRAFT-VERSUS-HOST DISEASE AND INTESTINAL MORPHOLOGY

Billingham and Brent (1957) described a runting syndrome in neonatal mice which received allogeneic lymphocytes to which they were unresponsive and, unlike organ grafting, in this case the graft was reacting against the host.

Diarrhoea and wasting are predominant features (Reilly & Kirsner 1965, Slavin & Santos 1973). The small intestine as important target organ is altered and villus atrophy with crypt hyperplasia and necrosis of the mucosa may occur (Reilly & Kirsner 1965, Elson et al 1977). However, a GvHR induced by injection of parental cells in to adult unirradiated mice only leads to infiltration of the mucosa with lymphocytes, mast cells and other morphonuclear cells (Guy-Grand et al 1978, Mowat & Ferguson 1982).

Although cell mediated cytotoxicity is a conventional explanation for the tissue damage which occurs (Singh, Sabadini & Sehon 1972) during a GvHR, 'enteropathic' lymphokine production has to be considered as an alternative way of mediating tissue damage (Elson et al 1977, MacDonald & Ferguson 1977, Mowat & Ferguson 1982). Local CMI responses in the intestine, on the one hand, have possibly a protective effect against pathogenetic organisms and, on the other hand, are also responsible for intestinal damage (Ferguson & Parrott 1973, Mowat & Ferguson 1982).

B. CYCLOPHOSPHAMIDE AND INDUCTION OF LOCAL CELL MEDIATED IMMUNE RESPONSES

It has been demonstrated that cyclophosphamide treatment before feeding ovalbumin induces a local CMI on challenge with the antigen at a later date (as assessed by intraepithelial lymphocyte infiltration,

increased crypt length and crypt cell production rates (Mowat & Ferguson 1981)). On the other hand, I have demonstrated that cyclophosphamide treatment abrogates systemic tolerance. In the work discussed in this chapter, I first demonstrate that I am familiar with the techniques used in the following experiments and that I can measure parameters of local mucosal CMI, for example, induced by a GvHR in neonatal mice. In addition, I have tested the validity of the experimental protocol by repeating key experiments reported in the literature (Mowat & Ferguson 1981). Based on the demonstration of various ways of abrogation of oral tolerance (see Chapter 4), I then investigated whether abrogation of systemic tolerance does subsequently lead to local CMI on intestinal antigen challenge.

C. INDUCTION OF MUCOSAL CELL MEDIATED IMMUNE RESPONSES BY A GRAFT-VERSUS-HOST DISEASE IN IMMATURE ANIMALS

I. Experimental protocol and results

Neonatal BDF_1 mice of both sexes received 1×10^7 parental C57BL/6 spleen cells intraperitoneally, whereas age matched controls received 1×10^7 syngeneic spleen cells in RPMI 1640 (Table 7.1).

1. General health and development

All animals survived the initial handling. All animals were weighed every other day and showed a normal development until about 10 days after GvHR induction. Animals having received an intraperitoneal injection of allogeneic cells developed signs of overt disease indicated by five deaths (Figure 7.2). Surviving animals were lethargic, developed diarrhoea and failed to gain weight satisfactorily ($p<0.001$).

Table 7.1

Induction of graft-versus-host reaction in neonatal and adult mice

EXPERIMENTAL PROTOCOL:

Neonatal animals (BDF_1, males and females):

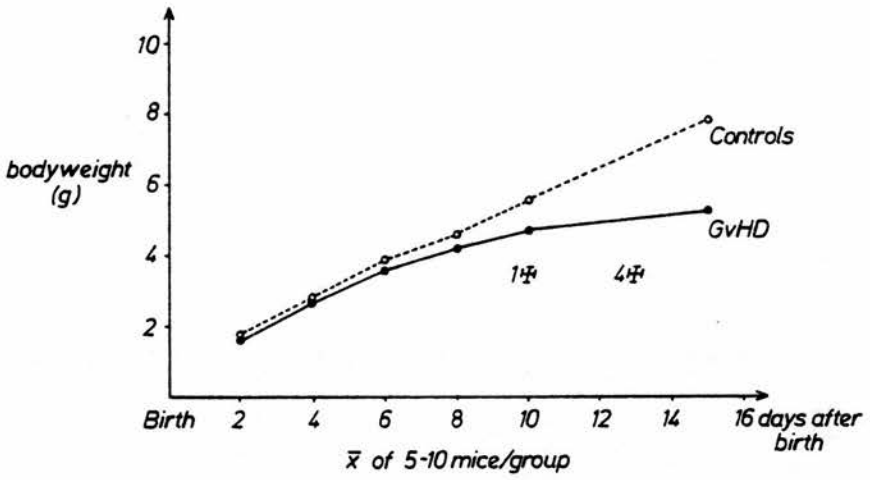
1st day of life:	intraperitoneal injection of 1×10^7 C57BL/6 spleen cells or intraperitoneal injection of 1×10^7 BDF_1 spleen cells in controls
15th day of life:	body weights spleen weights morphometry histology

Adult animals (aged 6 weeks) (CBA x BALB/c)F_1 (males and females):

1st day of experiment:	intraperitoneal injection of 6×10^7 CBA spleen cells or intraperitoneal injection of 6×10^7 (CBA x BALB/c)F_1 spleen cells in controls
13th day of experiment:	body weights spleen weights morphometry histology

All spleen cells injected were of female donor animals.

Effects of i.p. injection of syngeneic (BDF$_1$ ○) or semiallogeneic (C57 BL/6 ●) spleen cells on the weight gain of neonatal BDF$_1$ mice



7.2 **Effects of intraperitoneal injection of syngeneic (BDF_1) or semi-allogeneic (C57BL/6) spleen cells on the weight gain of neonatal BDF_1 mice**

Crosses indicate deaths of animals after induction of GvHD.

2. Macroscopic examination on sacrifice

On sacrifice, the stomachs of the animals with overt GvHD were severely distended with milk and the remaining intestine was pale, rigid and fragile and distended with watery, yellowish, faecal material. The spleens appeared to be enlarged.

Control animals which received syngeneic cells remained normal.

3. Spleen Index

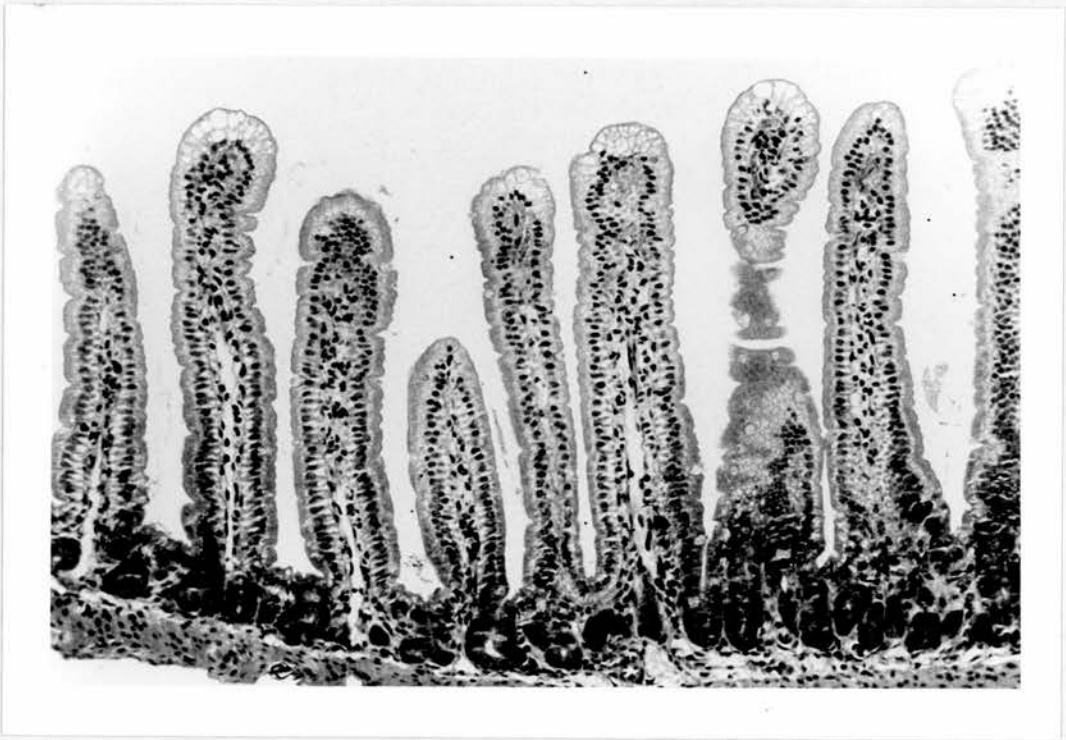
The visual impression of a GvHD on clinical grounds and by spleen enlargement was substantiated by a Spleen Index of 1.64 which indicated a significant splenic hypertrophy.

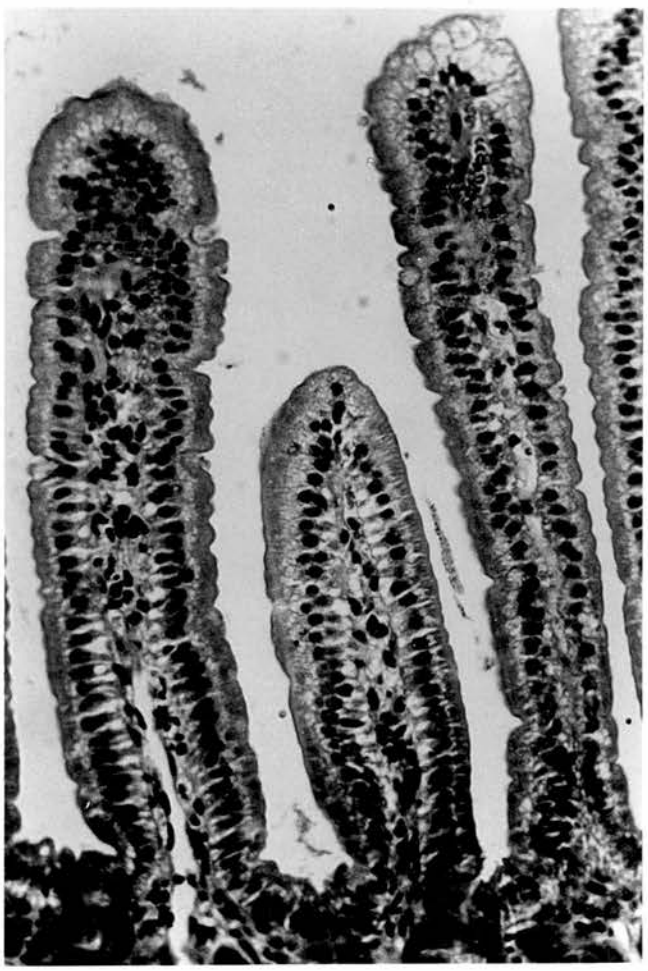
4. Histological examination

The histology of a two week old mouse with GvHD is shown in Figure 7.3b and compared with a littermate control. The striking features in the diseased intestine are grossly elongated crypts, shortened villi with some swollen tips and a general oedema. There is no acute inflammatory infiltration and the lamina propria is only sparsely populated. The amount of mucus seems to be increased and there is some epithelial cell extrusion and cell loss via the villus tips. Contrasting these findings, the normal neonatal intestine shows a thin mucosa with short crypts and long, rather regular, villi (Figure 7.3a).

(a) Intraepithelial lymphocyte infiltration

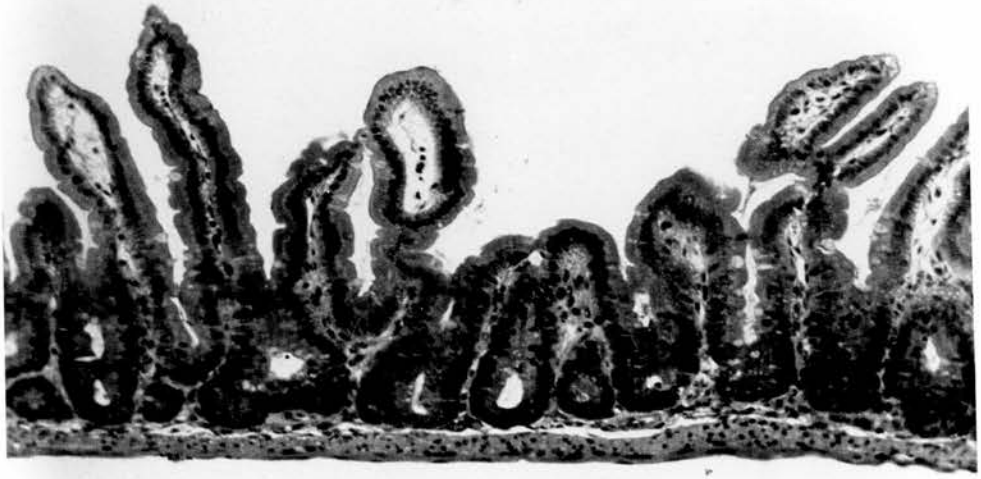
As anticipated, intraepithelial lymphocyte counts of mice injected with syngeneic cells did not differ from the non-injected age matched controls and the mean count per 100 epithelial cells of these animals ($n = 10$) was 0.55 ± 0.18 (Figure 7.4, $\bar{x} \pm SD$). These numbers differ significantly from those obtained in adult animals which at six weeks

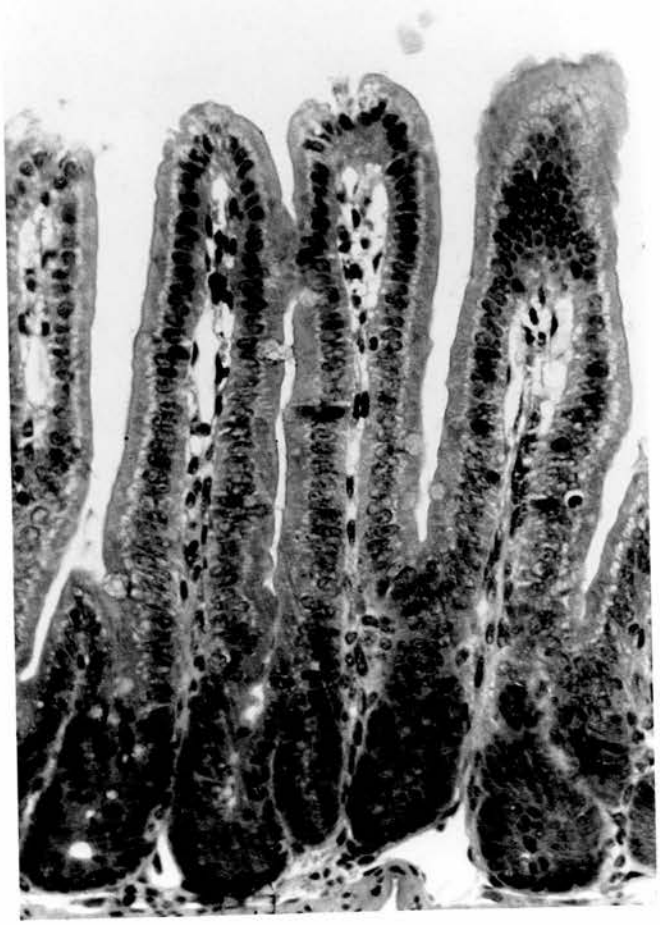




7.3a **Normal jejunal histolog of a 15 day old BDF_1 mouse**

Note the long finger-shaped villi and short crypts (magnification: top x160, bottom x320)





7.3b **Intestinal damage due to graft-versus-host disease in a 15 day old BDF_1 mouse (jejunum)**

Note the irregular villus pattern with elongated crypts, shortened villi and a general oedema of the mucosa. The amount of mucus seems to be increased and there is increased cell loss via the villus tips. The lamina propria is only sparsely populated (magnification top x160, bottom x320).

had a count of 10.4 $\pm$ 1.3 per 100 epithelial cells. Neonatal animals however which received allogeneic cells showed a signficiant increase of intraepithelial lymphocytes in their mucosa and the count was raised to 3.1 $\pm$ 0.5 ($p<0.01$, $n = 5$, $\bar{x} \pm$ SD).

(b) Mucosal mast cells

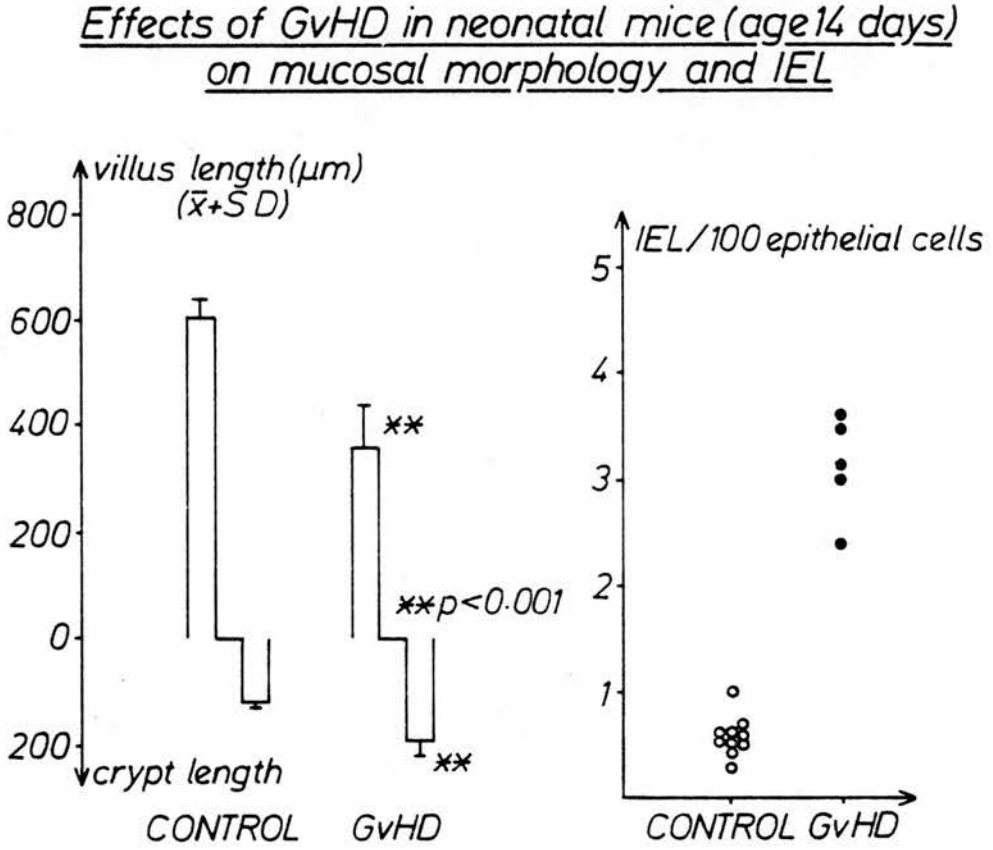
Very few mucosal mast cells could be detected in the mucosa under oil immersion (x1000). The distribution of these cells in GvHR was not obviously different from controls. Because of the scarcity of mucosal mast cells resulting in a wide scatter, no significant differences were found. The overall mean was 39 mast cells/mm^2 ($n = 12$).

(c) Mucosal morphology

Control animals which received syngeneic cells showed no changes in their villus heights or crypt lengths (villus height: 606 $\pm$ 38 µm, crypt length 118.2 $\pm$ 4.5 µm, $\bar{x} \pm$ SD) and the villus/crypt ratio was normal at 5.1. Animals with GvHR had a reduced villus height of 355 $\pm$ 80 µm and in increase in crypt length of 185 $\pm$ 30 µm, thus reducing the villus/crypt ratio to 1.8 ($p<0.001$) (Figure 7.4).

II. Comment

By injection of parental cells into neonatal animals at the first day of life, I was able to induce overt GvHD with significant changes in intraepithelial lymphocyte infiltration and mucosal morphology. These results complement the findings of Mowat and Ferguson (1982) who reported a GvHR in neonatal animals which were injected five to seven days after birth. In their experiments, induction of a GvHR in five to seven day old mice did not cause villus atrophy or overt GvHD and they reported a normal weight gain. It has however to be stressed that they



7.4 **Effects of graft-versus-host disease in neonatal mice on mucosal morphology and intraepithelial lymphocyte infiltration**

used a different strain combination (CBA x BALB/c) F_1 and injected the cells at a later date; the differences reported here are likely to be due to these facts.

D. INDUCTION AND ASSESSMENT OF GRAFT-VERSUS-HOST REACTION IN ADULT ANIMALS

Adult CBA x BALB/c animals received 6 x 10^7 semi-allogeneic spleen cells according to the experimental protocol and controls were treated as indicated in Table 7.1.

I. General health and development

All animals remained healthy during the time of the experiment, they showed a steady weight gain and in particular did not develop any signs of diarrhoea.

II. Spleen Index

Animals which were sacrificed on the 12th day after induction GvHR showed a Spleen Index of 2.25 for females and 2.20 for males, indicating a signficant splenic hypertrophy. Splenic hypertrophy as assessed by the Spleen Index was still present 50 days after GvHR induction with 1.78 for females and 1.4 for male CBA x BALB/c mice.

III. Histological findings

1. Intraepithelial lymphocyte infiltration

Examination of histological sections revealed no pathological changes of mucosal architecture 50 days after GvHR. Intraepithelial lymphocyte infiltration as an additional measure of an ongoing CMI

still showed a significant increase in diseased animals (19.0 $\pm$ 1.23) compared to control animals (10.7 $\pm$ 0.33, $p<0.001$, $\bar{x} \pm SD$) (Figure 7.5).

2. Mucosal morphology

Mucosal morphology was unchanged (Figure 7.5).

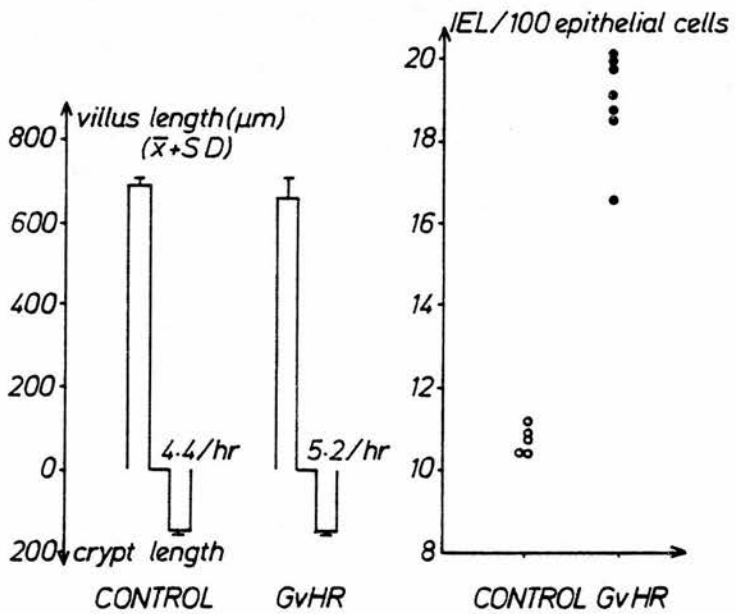
IV. **Comment**

Whereas injection of parental spleen cells into one day old neonates led to an overt GvHD disease with marked changes in mucosal morphology, injection of parental cells into adult F_1 animals induced a signficant increase in intraepithelial lymphocyte infiltration and no further mucosal abnormalities. These results were comparable to those obtained by other investigators in the same laboratory (Mowat 1981). By obtaining these results I demonstrated that I was familiar with the microdissection and intraepithelial lymphocyte counting techniques which have been used in the following experiments. Intraepithelial lymphocyte infiltration and a raised Spleen Index are the only features found after GvHR induction in adult animals of the above stated strain combination. Although these animals did not exhibit any signs of disease, splenic hypertrophy and intraepithelial lymphocyte infiltration can be demonstrated until 50 days after GvHR induction.

E. EFFECT OF CYCLOPHOSPHAMIDE ON INDUCTION OF MUCOSAL CELL MEDIATED IMMUNITY AFTER CONTINUOUS OVALBUMIN CHALLENGE

Induction of local mucosal CMI to a dietary antigen has been demonstrated after abrogation of systemic tolerance by cyclophosphamide (Mowat & Ferguson 1981). Before investigating the effects of a single

Effects of GvHR induced in 6-8 week old (CBA x BALB/c) F_1 mice on mucosal morphology and IEL (50 days after GvHR induction)



7.5 Effects of a graft-versus-host response induced in adult (CBA x BALB/c)F_1 mice on jejunal morphology and intraepithelial lymphocyte infiltration 50 days after induction

There was a significant increase in numbers ($p<0.001$) of intraepithelial lymphocytes in mice with GvHR after 50 days although mucosal morphology was unchanged.

low and high dose challenge on intestinal CMI I repeated the experiment in BALB/c and BDF_1 animals.

I. **The experimental protocol** is outlined in Figure 7.6 and was followed in all subsequent experiments. Female mice of both strains were injected with cyclophosphamide two days prior to an ovalbumin or saline feed, rested for 28 days and then challenged with 0.1 mg ovalbumin/day in their drinking water.

II. **Results in BALB/c mice:**

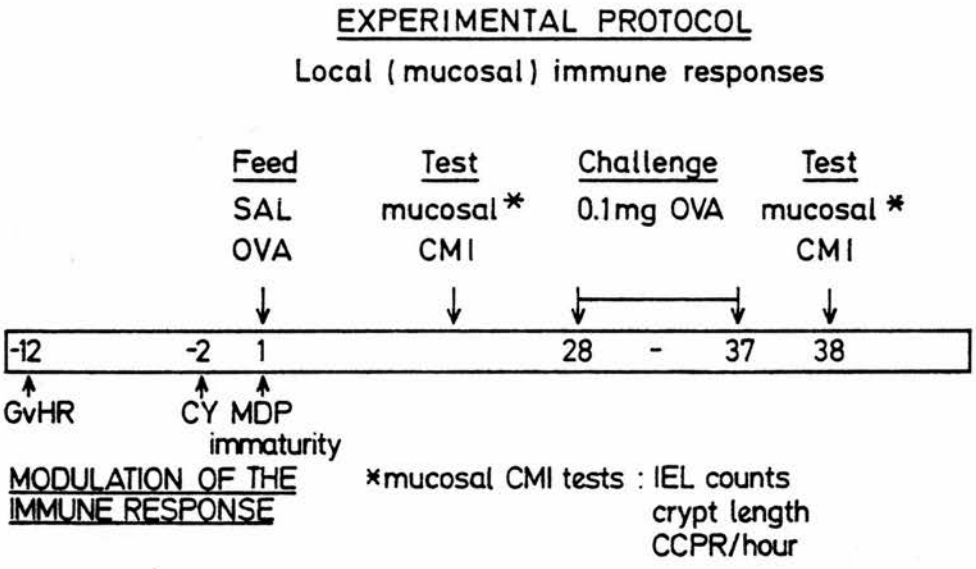
All animals remained healthy throughout the experiments.

1. Intraepithelial lymphocytes: Mice fed saline and then challenged had normal intraepithelial lymphocyte numbers (10.3 ± 0.4) whereas those fed ovalbumin before challenge had an increased lymphocyte count of 19.6 ± 1.8, $\bar{x} \pm$ SD, $p<0.01$) (Figure 7.7).

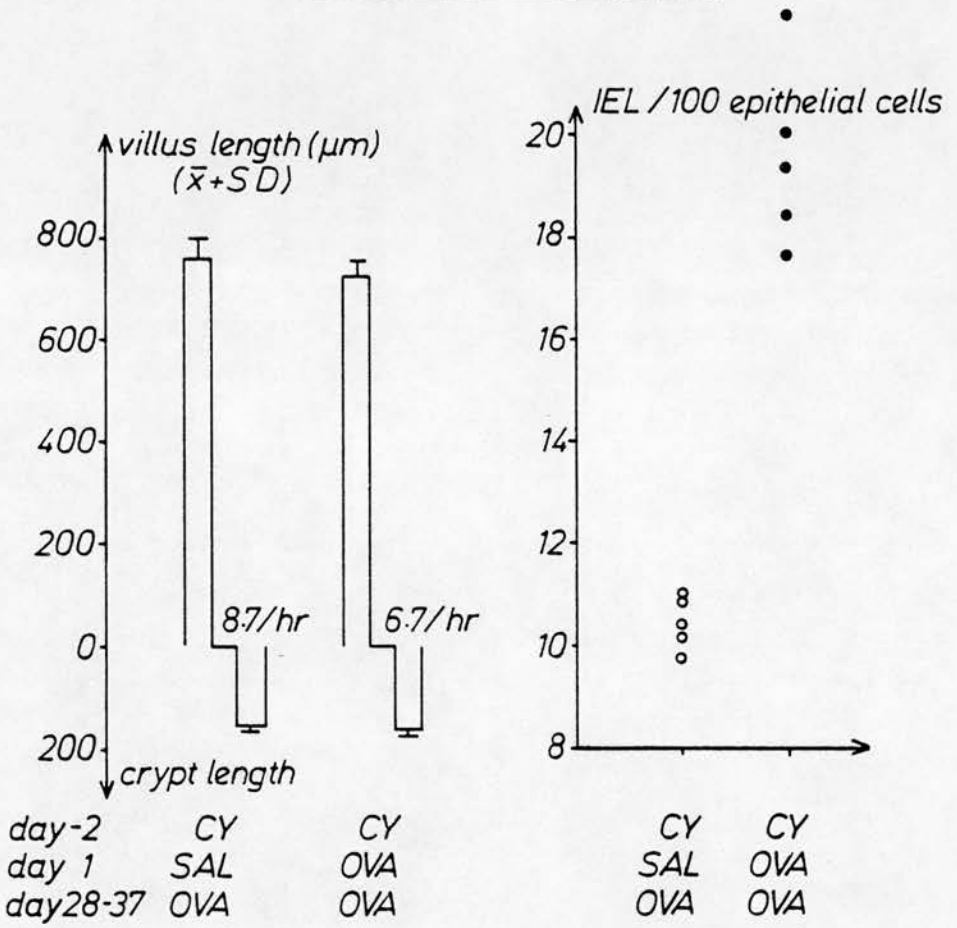
2. Mucosal mast cells: Mucosal mast cells were not a major part of intraepithelial or lamina propria cells in these animals. Their numbers varied from 0-2 per microscopic (x400) field, i.e. from 0-23/mm^2 of mucosa.

3. Mucosal morphology

BALB/c mice usually have long finger shaped villi of rather uniform length. Experimental animals having received ovalbumin before challenge displayed unaltered mucosal architecture with a villus length of 727.3 ± 33.8 µm, a crypt length of 159.1 ± 3.9 µm and a crypt cell production rate of 6.7/hour ($r = 0.940$) when compared to 756.2 ± 44.5 µm, 160.0 ± 3.8 µm, and a crypt cell production rate of 8.7/hour ($r = 0.987$) as seen in control animals (Figure 7.7).



7.6 **Experimental protocol for the investigation of local mucosal immune responses**



7.7 **Induction of a local mucosal cell mediated immune response to ovalbumin after cyclophosphamide treatment in adult BALB/c mice**

Mice which were treated with cyclophosphamide and then fed and challenged with ovalbumin developed a significant increase in intraepithelial lymphocyte numbers ($p<0.01$).

III. **Results in BDF_1 mice:**

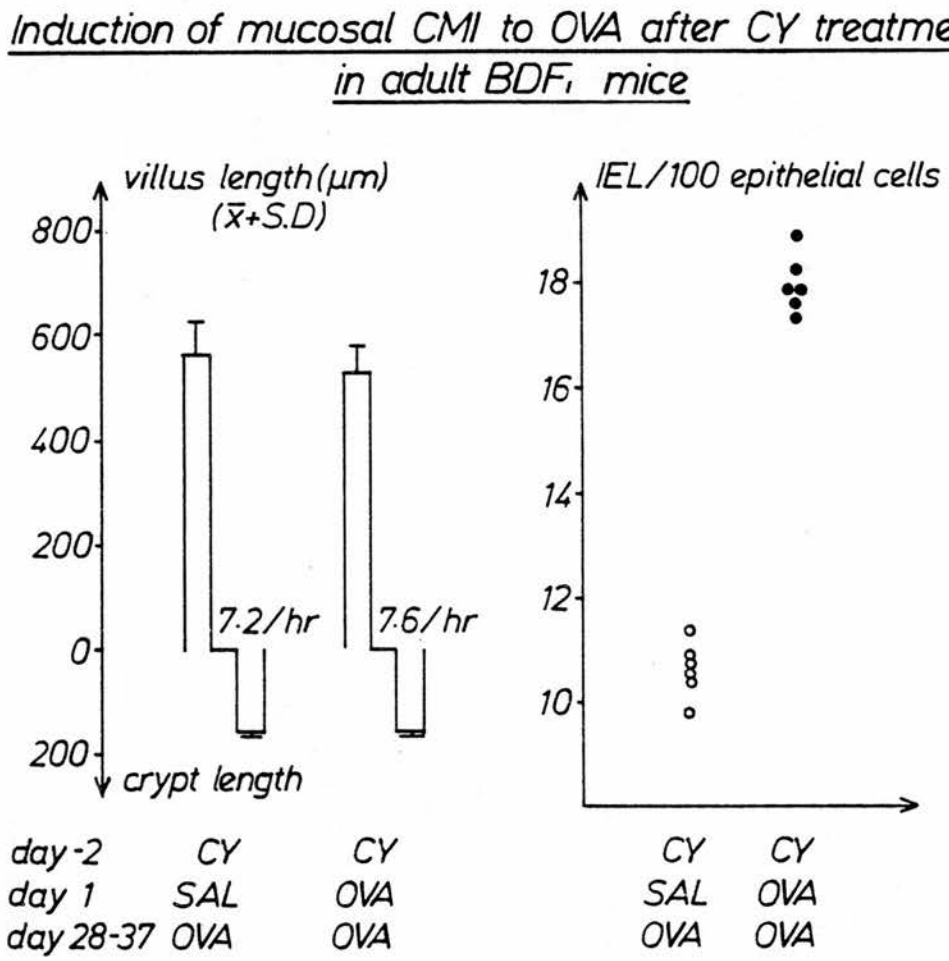
1. Intraepithelial lymphocyte counts: Mice fed water before ovalbumin challenge had an intraepithelial lymphocyte count of 10.6 ± 0.5 compared to 18.0 ± 0.6 ($p<0.01$) found in animals which were fed ovalbumin before the challenge (Figure 7.8).

2. Mucosal mast cells: There was no change in the usual low mast cell count of the lamina propria($0-20/mm^2$). There was no increase in granulated intraepithelial lymphocytes or 'atypical' intraepithelial mast cells.

3. Mucosal morphology: The villi of BDF_1 mice were generally shorter and their length more variable than those of BALB/c animals. Villus length of the control group was 564 ± 71 μm compared to mice which were fed ovalbumin 528 ± 56 μm (pNS). There also were no differences in either crypt lengths (153 ± 4.8 μm vs. 158.7 ± 5 μm) or crypt cell production rates which reached 7.2/hour ($r = 0.93$) compared to 7.6/hour ($r = 0.99$) (Figure 7.8).

IV. **Comment**

A common and consistent feature in all these experiments was the signficant rise in intraepithelial lymphocytes after cyclophosphamide pretreatment and oral sensitisation. These results demonstrate that changes in mucosal architecture such as increased crypt lengths or decreased villus heights are not the inevitable consequence of a local CMI as assessed by increased intraepithelial lymphocyte numbers. By using a recently established protocol for induction of a local CMI (Mowat & Ferguson 1981), I failed to observe signicant morphological



7.8 **Induction of a local cell mediated immune response to ovalbumin after cyclophosphamide treatment in adult BDF_1 mice**

BDF_1 mice developed an increase in intraepithelial lymphocyte numbers after ovalbumin challenge ($p<0.01$) similar to that observed in BALB/c mice (7.7).

changes, for example, in crypt length and in crypt cell production rates.

These experiments have been repeated several times with virtual identical results; a significant rise in intraepithelial lymphocytes proving a reliable marker of a local CMI. The reasons for the inability to demonstrate significant morphological changes remain speculative. Genetic differences in the immune responsiveness cannot be ruled out and a genetic difference, for example, in immune exclusion, has been recently demonstrated (Stokes, Swarbrick & Soothill 1983). Furthermore, a change in the adjuvanticity of the gastrointestinal flora of the animal stock could have caused these different responses. In support of this hypothesis, it has recently been reported that lipopolysaccharides play an important regulatory role during induction of oral tolerance to sheep erythrocytes (Michalek, Kiyono, Wannemuehler, Mosteller & McGhee 1982, Wannemuehler, Kiyono, Babb, Michalek & McGhee 1982) and further studies are needed to resolve these interesting hypotheses.

F. EFFECTS OF CYCLOPHOSPHAMIDE PRETREATMENT AND A SINGLE OVALBUMIN CHALLENGE ON INDUCTION OF LOCAL CELL MEDIATED IMMUNITY

A continuous 10 day challenge has been shown to increase intraepithelial lymphocyte numbers in previously sensitised animals. The following experiments were designed to investigate whether the responses of the intraepithelial lymphocytes and those concerning intestinal morphology were qualitatively and quantitively dependent on the amount of ovalbumin used for a single challenge and on the time lapse between ovalbumin administration and sacrifice.

I. Experimental protocol and results:

The general experimental protocol (Figure 7.6) was followed and the challenge was 1/10th of the long term challenge dose (100 µg ovalbumin) or a repeat of the initial sensitising dose of 25 mg ovalbumin.

1. Intraepithelial lymphocytes

The individual results are depicted in Figures 7.9 and 7.10 and show that even as little as 100 µg ovalbumin and as much as 25 mg ovalbumin intragastrically administered are sufficient in sensitised animals to lead to an increase of intraepithelial lymphocyte numbers 24-30 hours after challenge (17.8 ± 1.0 and 17.5 ± 1.2) compared to a mean count of 10.8 ± 0.5/100 epithelial cells of four non-sensitised control groups ($p<.01$).

2. Mucosal mast cells

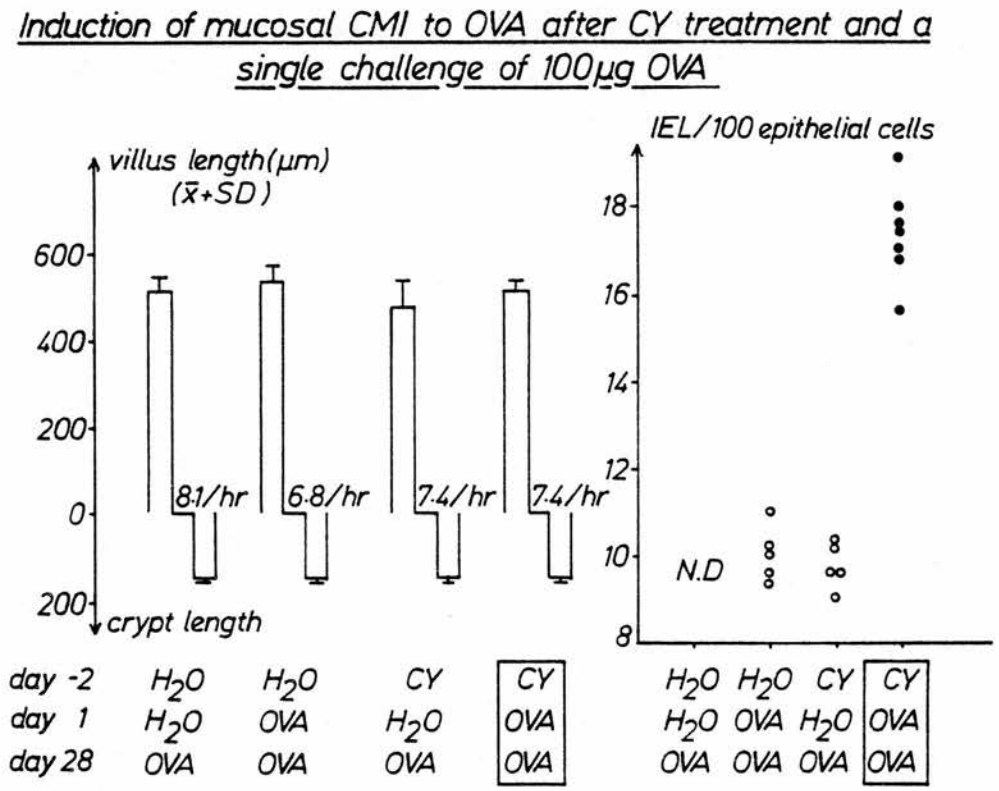
Mast cell infiltration was sparse and no changes in mucosal mast cells or atypical intraepithelial mast cells were observed.

3. Mucosal morphology

The results of all groups are shown in Figures 7.9 and 7.10. There were no significant changes in villus or crypt lengths or crypt cell production rates.

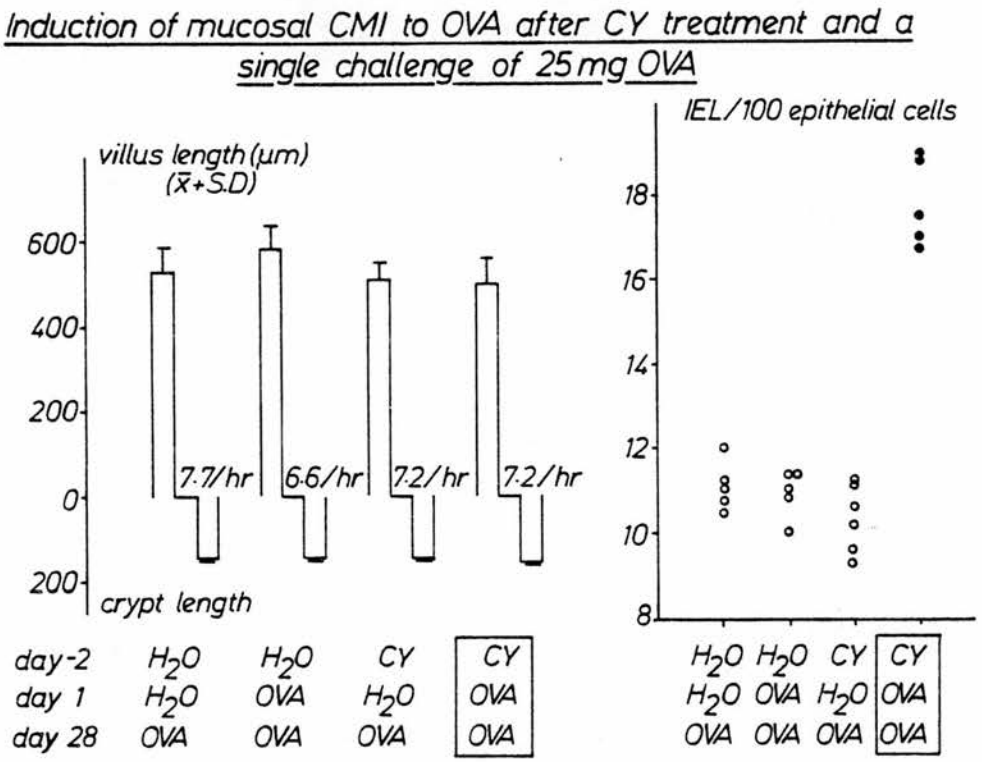
II. Comment

A consistent feature in this experiment was an increase in intraepithelial lymphocytes and this seems to be the most reliable, early marker of a local CMI. A rapid and significant increase in intraepithelial lymphocytes after ovalbumin challenge was observed even after 24 hours. The lymphocytic response could be elicited by feeding



7.9 **Induction of mucosal cell mediated immunity to ovalbumin after cyclophosphamide treatment followed by a single challenge of 100 µg ovalbumin**

Those conditions which gave rise to a significant infiltration of intraepithelial lymphocytes ($p<0.01$) are outlined. It can be seen that other mucosal parameters were not affected (N.D. = not done).



7.10 **Induction of mucosal cell mediated immunity to ovalbumin after cyclophosphamide treatment followed by a single challenge of 25 mg ovalbumin**

Those conditions which gave rise to a significant intraepithelial lymphocyte infiltration ($p<0.01$) are outlined.

100 µg or 25 mg of ovalbumin. A similar rapid rise in intraepithelial lymphocytes within 24 hours has been observed by Mowat and Ferguson (1982) when they induced a GvHR in one week old mice. My experiments however show that, following an established protocol for induction of local CMI by cyclophosphamide, additional characteristic features of a local CMI such as an increase in crypt length and in cell turnover and/or a reduction in villus lengths are not always part of this intestinal immune response. These results further indicate that the gut associated lymphoid tissue exerts a substantial immunoregulatory control in preventing morphological changes and that the intestinal damage seen in local CMI reactions is not an 'all or nothing' phenomenon but can proceed step by step, a rise in intraepithelial lymphocytes being the first visible histological change.

G. EFFECTS OF GRAFT-VERSUS-HOST RESPONSE ON INDUCTION OF LOCAL CELL MEDIATED IMMUNITY TO OVALBUMIN

It was demonstrated earlier (Chapter 4) that a low grade GvHR partially reversed systemic tolerance when the antigen was fed during the early (proliferative) phase. The following experiments were designed to investigate whether induction of an additional local CMI to a new antigen (ovalbumin) would amplify the histological changes demonstrated at the beginning of this chapter.

I. **Experimental protocol and results**

The general protocol (Figure 7.6) was followed; (CBAxBALB/c) F_1 mice were fed ovalbumin 12 days after induction of a GvHR, rested for 28 days and then challenged with ovalbumin in their drinking water.

1. The general health of all animals was unaffected throughout the entire experiment.

2. Spleen Index

The mean Spleen Index was 1.6 (female mice 1.78, male mice 1.4), 50 days after the injection of semi-allogeneic cells and indicated an effective induction of a GvHR.

3. Histology

(a) Intraepithelial lymphocyte numbers

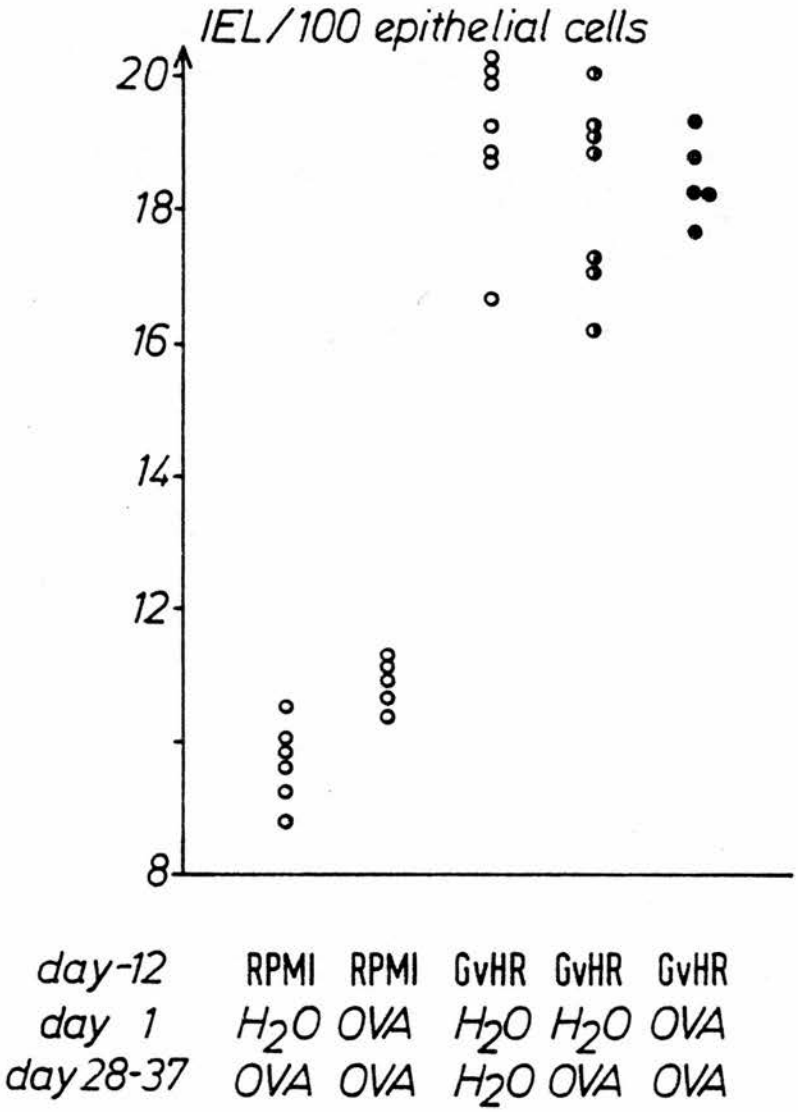
The results of these counts are depicted in Figure 7.11a. All experimental groups with GvHR had increased counts regardless of whether they consisted of water fed and water challenged controls (19.0 $\pm$ 1.23) of water fed and ovalbumin challenged (18.9 $\pm$ 2.3) or of ovalbumin sensitised and challenged animals (18.4 $\pm$ 0.7, intraepithelial lymphocytes/100 epithelial cells, $\bar{x} \pm$ SD).

Control animals had significantly lower ($p<0.01$) counts of intraepithelial lymphocytes (10.9 $\pm$ 0.24; 9.1 $\pm$ 0.43, $\bar{x} \pm$ SD). A striking feature was the finding of a high proportion of intraepithelial cells being granulated, the percentage of which was unrelated to the initial treatment.

(b) Mucosal mast cells and atypical granulated cells

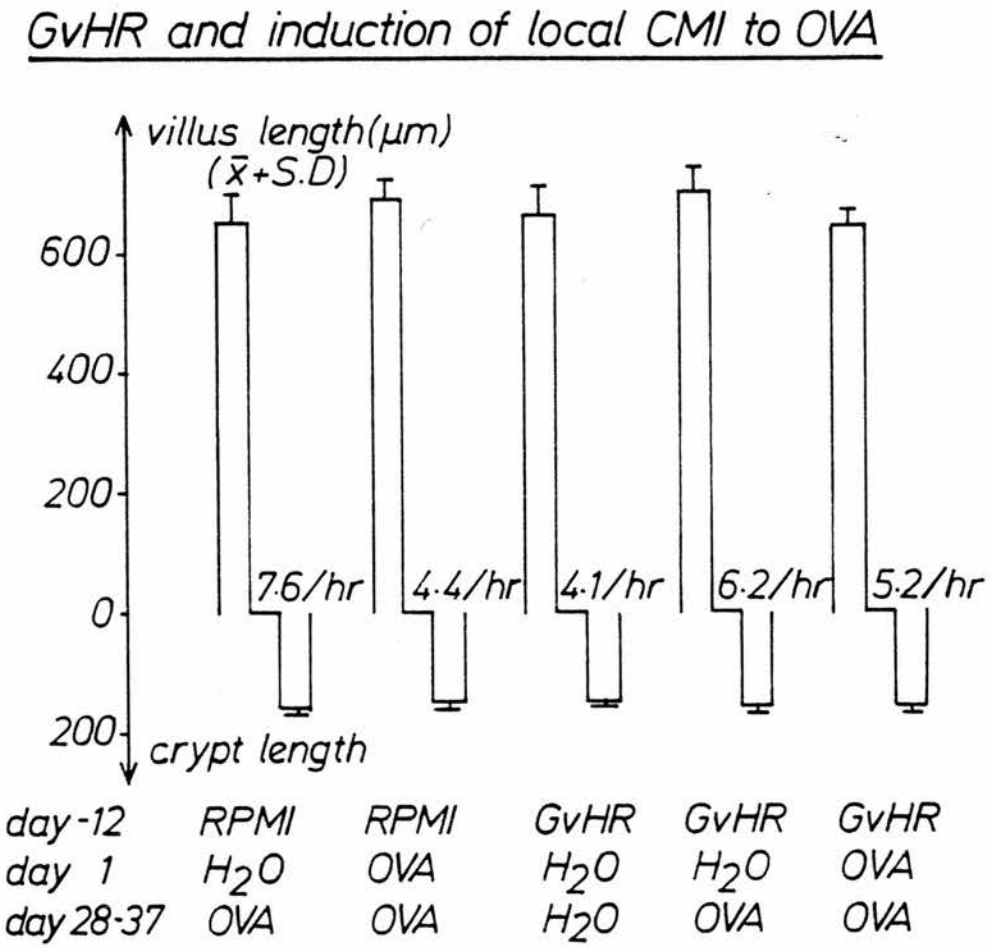
A striking feature of all (CBA x BALB/c) F_1 animals was the abundance of lamina propria and intraepithelial granulated cells, which comprised normal mucosal mast cells, atypical intraepithelial mast cells and intraepithelial granulated lymphocytes. Nearly all intraepithelial lymphocytes were found to be granulated and the mean ratio of non-granulated/granulated lymphocytes in animals with GvHR was 1.14:1 compared to 1.02:1 (Table 7.12). None or negligible numbers of

GvHR and induction of local CMI to OVA



7.11a **Effects of a graft-versus-host response on the induction of a local cell mediated immune reaction to ovalbumin**

A GvHR led to a rise in intraepithelial lymphocyte numbers ($p<0.01$) and this response was not increased by oral immunisations with ovalbumin.



7.11b **Effects of a graft-versus-host response on the induction of local cell mediated immunity to ovalbumin**

There were no differences in mucosal morphology or crypt cell production rates.

Table 7.12 **Granulated and non-granulated intraepithelial lymphocytes in different strains of mice**

Experimental group (CBA x BALB/c) F_1	Intraepithelial cells/100 epithelial cells: Total IEL count ($\bar{x} \pm SD$)	Granulated IEL ($\bar{x} \pm SD$)	Total IEL/ granulated IE cells	Granulated cells/mm^2 ($\bar{x} \pm SD$)
GvHR/SAL fed/OVA challenge	18.9 ± 2.3	16.6 ± 1.34	1.14	327 ± 42
GvHR/OVA fed/OVA challenge	18.4 ± 0.68	17.5 ± 0.78	1.05	326 ± 20
GvHR/SAL fed/water challenge	19.0 ± 1.23	15.6 ± 0.41	1.22	307 ± 23
RPMI/SAL fed/OVA challenge	9.2 ± 0.43	9.1 ± 0.27	1.01	313 ± 34
RPMI/OVA fed/OVA challenge	10.9 ± 0.24	10.6 ± 0.21	1.03	363 ± 41
CBA (females)	12.0 ± 0.72	0 - 0.002	00	10.3 ± 3.6
BALB/c (males)	9.95 ± 1.0	0 - 0.001	00	17.3 ± 0.95

granulated intraepithelial lymphocytes were seen in the parental strains (CBA females: 0.2 - 0.02/100 epithelial cells; BALB/c males: 0). Counting the numbers of all astra blue positive cells/mm^2 mucosa, GvHR animals showed 330 $\pm$ 11.3/mm^2 compared 338 $\pm$ 35.4 in control animals ($\bar{x} \pm$ SD) (Table 7.12). Cell numbers in the parental strains were considerably lower (CBA females 10.3 $\pm$ 3.6; BALB/c males 17.3 $\pm$ 0.95/mm^2 mucosa).

(c) Mucosal morphology

Villus and crypt lengths as well as crypt cell production rates did not differ in experimental and control groups as is illustrated in Figure 7.11b. Villus lengths in the GvHR group were 666 $\pm$ 28.5 µm (n = 20) compared to 670 $\pm$ 26.9 µm (n = 11). The crypt length was 153.8 $\pm$ 4.1 µm in the GvHR group vs. 157.4 $\pm$ 7.6 µm in control animals. The range of crypt cell production rates was 4.1 - 6.2/hour in animals with GvHR compared to 4.4 - 7.6/hour in control mice. They did not differ significantly.

II. Comment

Induction of GvHR in adult mice led to an increase in the Spleen Index and to a rise of intraepithelial lymphocytes without altering the mucosal architecture as assesed by light microscopy. Attempts to further sensitise animals for a local CMI to ovalbumin during an ongoing CMI response did not lead to a further increase of intraepithelial lymphocytes or to induction of additional mucosal changes in (CBA x BALB/c) F_1 animals. A striking feature of this hybrid generation was the detection of a high proportion of intraepithelial lymphocytes demonstrating astra blue positive granules which were well above the

numbers found in both parental strains. This feature was not seen in the (C57BL/6 x DBA2) F_1 generation. Thus in the experiments described above, a high number of granulated cells was not positively correlated with a GvHR but rather with the strain of mice investigated.

H. EFFECTS OF MURAMYL-DIPEPTIDE ON THE INDUCTION OF LOCAL INTESTINAL CELL MEDIATED IMMUNITY TO OVALBUMIN

It has been demonstrated in Chapter 4 that the systemic immune responses to a feed of ovalbumin could be altered by intraperitoneal administration of muramyl-dipeptide at the time of antigen feeding and that oral tolerance was abrogated by this treatment. In the following experiments, I wanted to investigate whether the reversal of systemic tolerance for CMI could have led - at the same time - to a sensitisation of the gut associated local immune system.

I. Experimental protocol and results

The general experimental protocol (Figure 7.6) was followed and 50 µg muramyl-dipeptide in saline were injected intraperitoneally immediately before feeding ovalbumin or saline to the animals. After a rest of 28 days all animals were challenged with 0.1 mg ovalbumin/day for 10 days.

1. Intraepithelial lymphcytes

All animals which had been injected saline and fed saline or ovalbumin (10.7 ± 1.6; 10.1 ± 0.5, $\bar{x} \pm SD$) or injected muramyl-dipeptide and fed saline (10.5 ± 1.0, $\bar{x} \pm SD$) had normal counts. However, mice which were injected with the adjuvant and later fed ovalbumin demonstrated a much increased number of intraepithelial lymphocytes,

18.7 ± 1.6 ($\bar{x}$ ± SD, $p<0.001$, Figure 7.13.).

2. Mucosal mast cells

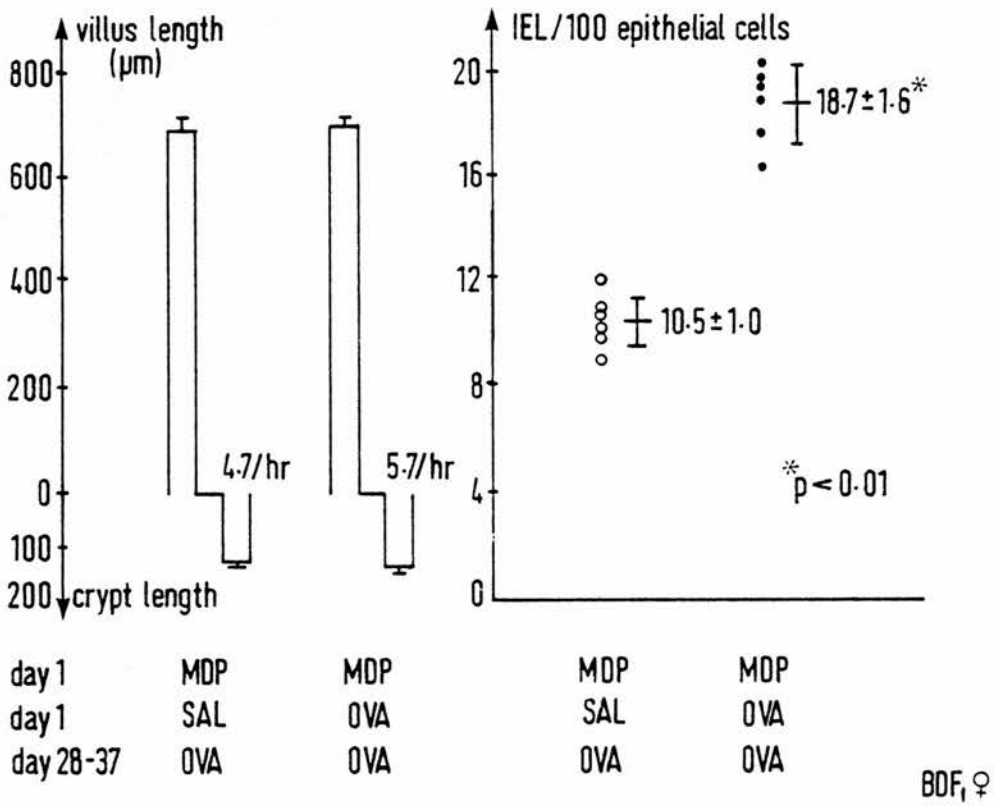
There was no change in mucosal mast cell infiltration (0-23/mm^2 mucosa).

3. Mucosal morphology

Since changes in mucosal morphology were never observed in saline fed and ovalbumin challenged mice, mucosal morphology was only investigated in direct comparison of adjuvant injected groups fed either saline or ovalbumin. There were no difference in these parameters, villus lengths were 689.3 ± 30.4 µm vs. 693.8 ± 21 µm, crypt lengths (134.4 ± 3.3 µm vs. 135.2 ± 4.0 µm) or crypt cell production rates (4.7/hour vs. 5.7/hour) (Figure 7.13).

II. **Comment**

A significant increase of intraepithelial lymphocytes after muramyl-dipeptide injection and ovalbumin sensitisation indicates an induction of a local CMI response. The adjuvant on its own did not raise the number of intraepithelial lymphocytes. It can be assumed that these effects are due to the immunostimulatory properties of muramyl-dipeptide (Leclerc et al 1979). The exact mechanisms and whether these effects are due to the action on macrophages or on a T-helper cell population (Löwy et al 1977) remains to be established and further investigation of the still hypothetical modes of action is beyond the scope of this thesis.



7.13 **Effects of immunomodulation by muramyl-dipeptide on the induction of mucosal cell mediated immunity to ovalbumin**

Immunomodulation by muramyl-dipeptide and oral immunisations with ovalbumin led to a rise in intraepithelial lymphocytes ($p<0.01$) while mucosal morphology remained unchanged.

I. EFFECTS OF A NEONATAL OVALBUMIN FEED ON LOCAL INTESTINAL CELL MEDIATED IMMUNE RESPONSES AFTER CHALLENGE

Digestive and/or immunological immaturity in the neonatal period have been implicated in explaining the mechanism which may sensitise mice for humoral antibody and CMI responses.

Clinical observations in humans and published direct or indirect evidence (Firer, Hosking & Hill 1981, Van Asperen, Kemp & Mellis 1983) indicate that introduction of a new dietary antigen in early life can lead to an intolerance to these antigens in later life. The following experiments were designed to address the question whether a neonatal sensitising feed of ovalbumin would also lead to induction of a local intestinal CMI on antigen re-exposure in later life. In doing so, this experimental protocol could serve as a pertinent model for the investigation of the mechanisms which govern the induction and expression of food related hypersensitivity diseases.

I. Experimental protocol and results

The experiments were conducted in two inbred mouse strains (CBA and BALB/c) and the general protocol (Figure 7.6) was followed:

1. CBA strain:

Because of the availability of this inbred strain in the Animal Unit of the Western General Hospital, Edinburgh, CBA mice were used for the first experiment of this series.

The control group consisted of age matched, non-saline fed mice under normal stock keeping conditions.

(a) Intraepithelial lymphocytes

There were no differences found in intraepithelial lymphocyte

infiltration in these two groups (11.7 ± 1.2 vs. 9.84 ± 0.7) (Figure 7.14).

(b) Mucosal mast cells

The lamina propria was sparsely infiltrated with mucosal mast cells (12.7 ± 3.6 vs. 10.3 ± 3.6 mm^2, $\bar{x}$ ± SD, pNS) and granulated cells were almost entirely confined to this site.

(c) Mucosal morphology

Despite unchanged numbers of intraepithelial lymphocytes, there was a clear increase in crypt length in the group which was challenged with ovalbumin (148.4 ± 10.8 μm vs. 170.4 ± 4.02 μm; $p<0.001$) with a slight reduction in villus height (647.5 ± 61 μm vs. 558 ± 67 μm, $p = 0.05$). The crypt cell production rate was slightly increased from 8.1/hour to 11.1/hour ($p<0.1$) (Figure 7.14).

2. BALB/c strain

(a) Intraepithelial lymphocytes

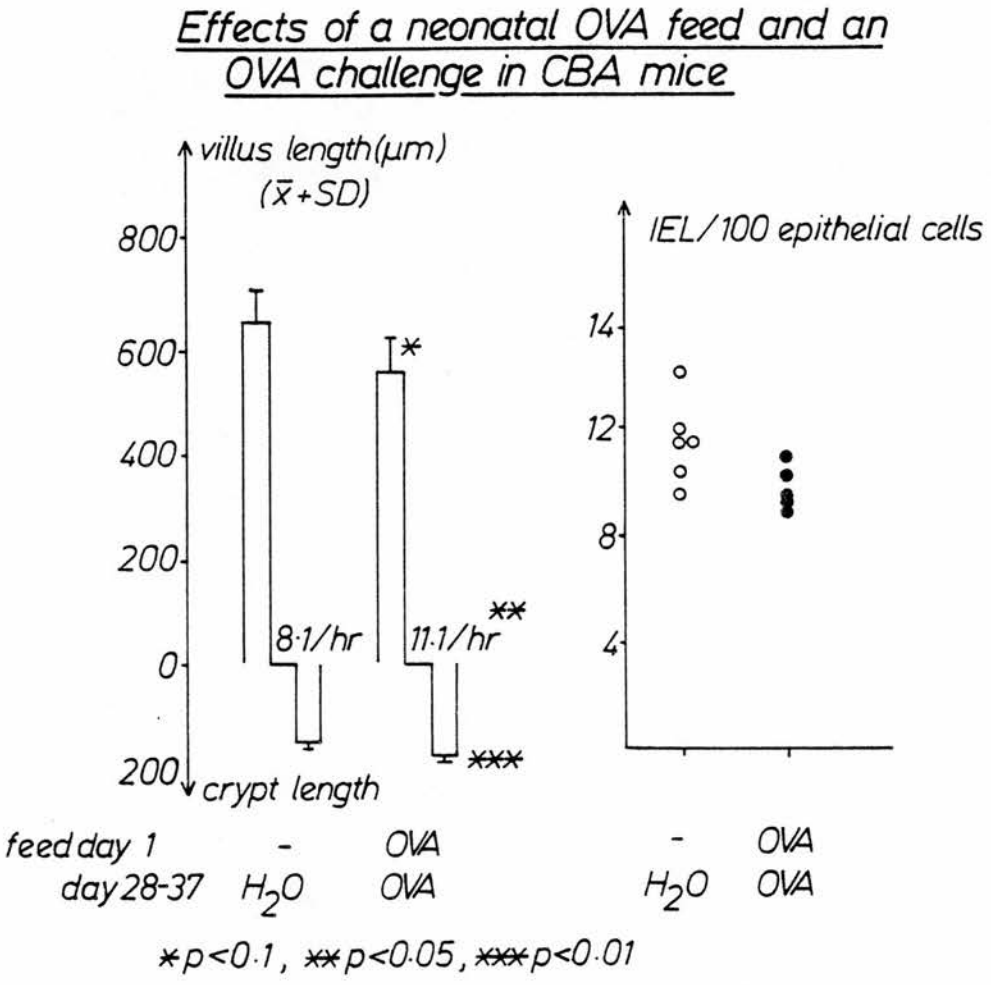
Animals which were fed ovalbumin on the first day of life and then challenged showed a striking increase of intraepithelial lymphocytes (9.3 ± 0.7 vs. 14.6 ± 2.5, $\bar{x}$ ± SD, $p<0.005$) (Figure 7.15). These experiments have been repeated with virtually identical results (11.2 ± 1.0 vs. 15.7 ± 2.1, $\bar{x}$ ± SD, $p<0.01$)

(b) Mucosal mast cells

Mucosal mast cells were found in a range between 10-40 cells/mm^2 mucosa without sizeable numbers of granulated intraepithelial cells in any of the experimental groups.

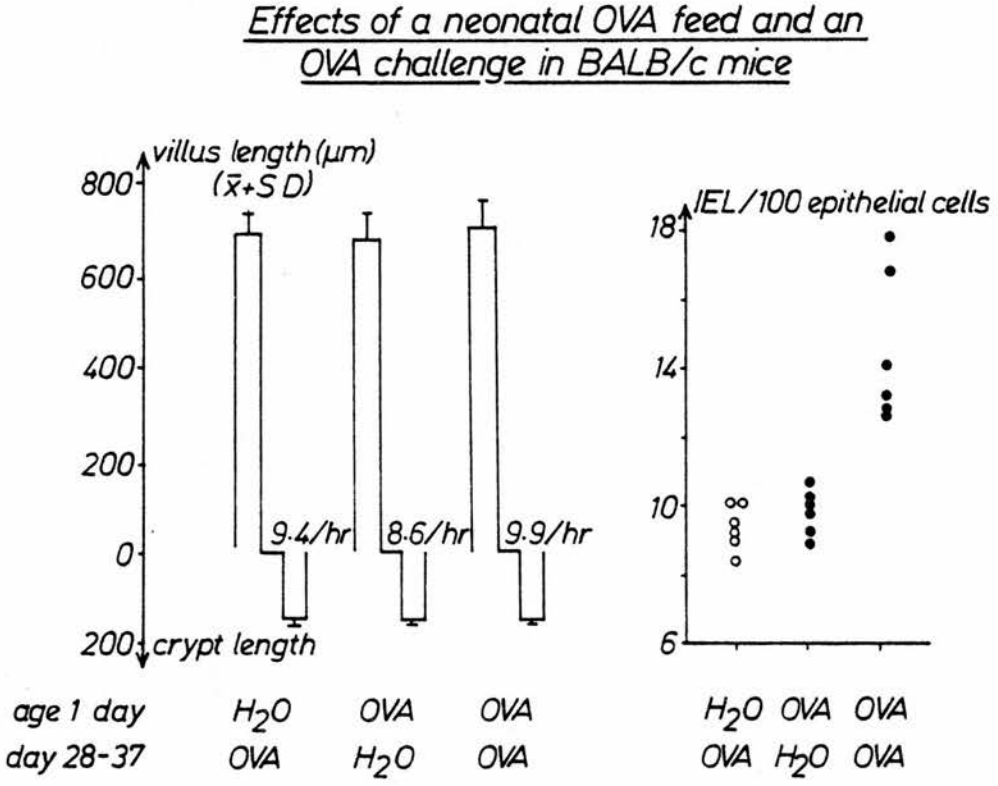
(c) Mucosal morphology

Assessment of morphological features in these groups did not show significant differences and is summarised below:



7.14 **Effects of a neonatal ovalbumin feed followed by an ovalbumin challenge in CBA mice**

An ovalbumin feed to one day old mice with a subsequent ovalbumin challenge led to a reduction in villus height ($p<0.01$), an increase in crypt length ($p<0.01$) and in the crypt cell production rate ($p<0.05$). There was no change in the numbers of intraepithelial lymphocytes.



7.15 **Effects of a neonatal ovalbumin feed followed by an ovalbumin challenge in BALB/c mice**

Feeding ovalbumin to one day old mice with a later ovalbumin challenge led to a significant increase of intraepithelial lymphocytes ($p<0.005$).

Day 1	saline fed:	villus height	688.3 ± 46.3 μm
		crypt length	148.0 ± 2.3 μm
		crypt cell	production rate 9.4/hr (r = 0.985)
Day 1	ovalbumin fed:	villus height	697.2 ± 64 μm
		crypt length	150.1 ± 1.6 μm
		crypt cell	production rate 9.9/hr (r = 0.940)

II. **Comment**

Both inbred mouse strains tested showed an effect of a sensitising feed of ovalbumin on the first day of life and responded with morphological (CBA) or cellular changes (BALB/c) on challenge with the antigen. CBA mice showed, however, no increase in intraepithelial lymphocyte numbers, a feature which was uniformly seen in BALB/c animals. The reason for this discrepancy cannot be satisfactorily explained by the results presented. The pilot experiments performed in the CBA strain however could be criticised on the grounds that the control animals were not sham fed on the first day of life and not challenged with ovalbumin in their drinking water, as had been done in all subsequent experiments (see Figure 7.6). It seems however unlikely that the effect of a sham feed in the neonatal period are still detectable in adult animals and that ovalbumin incorporated in the drinking water could have affected the mucosal architecture.

The experiments were repeated in BALB/c animals and an increase in intraepithelial lymphocytes was a consistent feature. Taking the presented results together, there is no doubt that an ovalbumin feed in the neonatal period sensitises for later local intestinal CMI responses.

The expression of the CMI however varies as discussed earlier and may be dependent on genetically different strains of mice, different gastrointestinal bacterial flora and may also depend on the timing of sensitisation and challenge procedures.

Chapter 8

GENERAL DISCUSSION

A. INTRODUCTION

The work of this thesis was designed to investigate factors which govern the induction and abrogation of orally induced tolerance and to investigate whether abrogation of systemic tolerance would lead to concomitant induction of local intestinal CMI. This overall objective has been achieved. I have been able to abrogate oral tolerance in mice by pharmacological means, such as injection of cyclophosphamide and muramyl-dipeptide prior to enteral antigen exposure. In addition, in these circumstances a local CMI could be induced on intestinal challenge with a protein antigen. Ovalbumin feeds during the proliferative phase of a GvHR did not induce oral tolerance. The role of the gut was investigated by transfer of serum from recently ovalbumin fed animals, by colonic administration, and by feeding ovalbumin to neonatal mice. The results of these experiments lend support to the hypothesis that antigen processing properties of the gut play a crucial role in the generation of oral tolerance and that humoral and CMI responses are controlled by different mechanisms.

A most important finding was that feeding a dietary antigen perinatally without further immunomodulation primed the animals <u>both</u> for systemic immunity <u>and</u> local intestinal CMI as measured by lymphocytic infiltration of the epithelium. I shall first discuss the work relating to the modulation of oral tolerance and to the role of the gut in generating systemic hyporesponsiveness. Special emphasis will be laid on the effects of age on oral tolerance induction. The induction of mucosal CMI after abrogation of oral tolerance will be discussed with reference to human pathophysiology and disease.

B. INDUCTION OF TOLERANCE BY FEEDING OVALBUMIN AND ITS MODULATION

After an initial exposure to a protein antigen by the oral route, specific secretory antibodies of the IgA class are produced in the gut mucosa. On the other hand, it is well documented that feeding of antigen also leads to a state of specific systemic hyporesponsiveness (oral tolerance) (Thomas & Parrott 1974, André et al 1975, Hanson et al 1977, Swarbrick, Stokes & Soothill 1979) and it has been demonstrated that a secretory antibody response and systemic tolerance may co-exist (Challacombe & Tomasi 1980). The immunoregulatory mechanisms responsible for this tolerant state remain controversial and the experiments were designed to gain more insight into the basic mechanisms in vivo. Immune suppression may be mediated by antibodies after sheep erythrocytes administration (Kagnoff 1978 a,b) or by circulating, presumably IgA immune complexes which are produced after oral immunisation (André et al 1975). Hanson, Vaz, Maia & Lynch (1979), however, have not been able to demonstrate immune suppression after transfer of serum of animals tolerised by ovalbumin feeds. In particular, cellular suppressor mechanisms appear to be important for maintaining the unresponsiveness after feeding ovalbumin (Miller & Hanson 1979, Challacombe & Tomasi 1980, Titus & Chiller 1981) or after feeding sheep erythrocytes (Kagnoff 1978b). Using sheep erythrocytes and feeding the lysed cells over 4-5 weeks in the drinking water, it was recently shown that there is evidence for induction of a feedback suppressor pathway in the spleens of these mice (McDonald 1982). In these experiments, antigen feeding appeared to activate a system in which Lyt 1+ cells can suppress immune responses by causing

normal T-cells to become T-suppressor/effector cells. Furthermore, this author failed to demonstrate the common, antigen-specific Lyt 2,3+ suppressor cells in the spleen after feeding. Suppressor cells in the Peyer's patches, generated after oral antigen encounter, have been demonstrated by Miller and Hanson (1979), by Ngan and Kind (1978) and Mattingly and Waksman (1978). The latter authors also described antigen specific helper and suppressor factors produced by spleen cells of rats fed sheep erythrocytes (Mattingly & Waksman 1980). It is too early to decide whether these discrepancies are due to experimental differences (soluble vs. particulate antigens, animal strains, feeding schedules) or whether the Lyt 2,3+ population in the Peyer's patch represents a non-circulating T-suppressor cell pool.

Induction of oral tolerance to ovalbumin in different inbred strains of mice

Induction of oral tolerance by feeding a single dose of ovalbumin to BALB/c, BDF_1 and CBA mice is a stable and antigen-specific phenomenon as demonstrated in Chapter 4 although one group of investigators reported the CBA strain to be more difficult to tolerise than other strains (Swarbrick 1979, Stokes et al 1983).

In trying to reduce animal numbers and costs as well as experimental time, I decided to immunise animals intradermally into the footpad. By choosing this route I was able to measure systemic humoral immunity and CMI in the same animal. The time interval from feeding to immunisation was reduced from 14 to seven days (Hanson & Miller 1979). By adapting the experimental protocol as outlined in Figure 4.3, I achieved the same degree of systemic hyporesponsiveness and was able to reduce the required animal numbers by half.

Persistence of tolerance

Monitoring the persistence of tolerance for both limbs of the immune response, I could demonstrate that the T and B-cell systems are affected differently. A similar observation has been made previously after intravenous injection of human gamma globulin (Chiller & Weigle 1971, Weigle, 1977). There were no reports of persistence of tolerance for delayed type hypersensitivity to ovalbumin in an in vivo system.

Tolerance for humoral immune responses has been shown to last for about 60 days (Vaz et al 1977, Ngan & Kind 1978, Challacombe & Tomasi 1980) and for over six months for CMI responses (antigen: sheep erythrocytes) (Kagnoff 1978 b). Measuring CMI responses in vivo, I showed that they were significantly suppressed for more than 17 months after the initial feed. Since most of the published reports did not investigate CMI responses in vivo it is not clear whether this phenomenon reflects differences in the assay systems (in vitro assays vs. in vivo footpad testing) or indicates a possible existence of a long lived immunoregulatory circuit for suppression of T_{DTH} effector cells. Cell transfer experiments would be an adequate way to answer these questions.

Modulation of oral tolerance:
Cyclophosphamide

It has been argued (Mowat & Ferguson 1981) that an important gut associated suppressor cell system exists to prevent the induction of local immune responses in the gut and gut associated lymphoid tissues in addition to producing systemic tolerance in response to oral antigen. In the mouse, suppressor T-cells are sensitive to certain doses of cyclophosphamide (Askenase, Hayden & Gershon 1975, Gill & Liew 1978) and

so cyclophosphamide may be expected to interfere with the generation of such cells following enteral antigen administration. The experiments using this drug were designed to investigate whether it might prevent the state of oral tolerance induced by a single feed. There is only a single report on the humoral antibody and CMI response in vivo after an ovalbumin feed to mice (Miller & Hanson 1979) and it is possible that the two limbs of the immune response may be dissociated. Evidence for this assumption has also been reported by Neveu and Borduas (1974) after parenteral antigen administration.

Parallel experiments were performed using 25 and 2 mg ovalbumin as tolerising procedures, the latter dose being close to the reported lower limit of tolerogenicity (Vaz et al 1977). Cyclophosphamide treatment prior to a feed of ovalbumin was used to investigate the role of suppressor cells and I have chosen a dose of cyclophosphamide within the range known to inhibit suppressor cells in the mouse when given two or three days before feeding. The two limbs of the immune response were affected differently by the two ovalbumin doses fed. CMI responses were more readily suppressed than humoral antibody responses (Figures 4.8 and 4.10). Furthermore, it is apparent from the work of others that differences exist even within the humoral reponse itself. There is evidence to suggest that IgE responses may be more readily suppressed than IgG responses (Ngan & Kind 1978). No such evidence exists at present on the relative susceptibilities of the IgM and IgG systems after orally induced hyporesponsiveness.

Careful interpretation of the antibody results obtained with and without addition of 2-mercaptoethanol (Figures 4.8 and 4.9) suggest that IgM production may be more readily suppressed by oral antigen

administration. In support of this observation, Kagnoff (1978b) has reported that IgM plaque forming cells are more susceptible to suppression by repeated feeding of sheep erythrocytes.

The results presented in Chapter 4 indicate a dissociation between CMI and the antibody responses in their susceptibility to induction of oral tolerance. CMI was markedly reduced by both doses of oral ovalbumin, despite unsuppressed antibody levels found in the same mice (2 mg ovalbumin). In this context the consequences of cyclophosphamide treatment in ovalbumin fed mice are also relevant. In mice fed 25 mg ovalbumin, cyclophosphamide treatment returned humoral and CMI responses to a level midway between control and tolerant mice. A different pattern however was seen in mice fed 2 mg ovalbumin. In this case, the markedly suppressed CMI responses were returned to control levels by the immunomodulating treatment. It has been suggested previously that oral tolerance may have multiple control mechanisms (Titus & Chiller 1981, Hanson et al 1979) and the findings support a system of oral tolerance dependent on several "suppressor" mechanisms activated by protein feeding. These may include induction of suppressor cells, functional deletion of helper T-cells and B-cell inhibition, the activation of these immunoregulatory circuits being dependent on the dose and quality of antigen presented to gut associated immune system.

While both suppressor cells or their products (Mattingly & Waksman 1978, Mattingly & Waksman 1980, Mattingly, Kaplan & Janeway 1980) and serum factors (André et al 1975, Kagnoff 1978a, Kagnoff 1980) have been reported in the unresponsive state following feeding of particulate antigens, it is likely that suppressor T-cells induced in the spleen and gut associated lymphoid tissues are responsible for tolerance to

protein antigens (Ngan & Kind 1978, Richman et al 1978, Miller & Hanson 1979, Titus & Chiller 1981). In addition, the induction of tolerance by feeding contact-sensitising agents to guinea pigs is related to the appearance of suppressor cells (Asherson et al. 1977) and is abrogated by cyclophosphamide treatment (Polak, Geleick & Turk 1975). Thus the tolerance of CMI responses after a low dose of ovalbumin which was fully sensitive to abrogation by cyclophosphamide in my experiments suggests that a suppressor cell mechanism is sufficient to account for the tolerance of CMI responses induced after small doses of oral protein.

Although, as indicated, there is ample evidence to support the view that this regime of cyclophosphamide treatment is acting by its effect on suppressor lymphocytes, it is important to consider the effects that this drug may have on the ability of the gut to process proteins. Experiments to address this question will be discussed later.

Effects of cyclophosphamide on oral tolerance in different strains of mice

Having demonstrated that phenotypically the systemic tolerance observed after feeding ovalbumin was similar in CBA, BALB/c and BDF_1 mice, the effects after cyclophosphamide treatment could still have been different. A report of Hanson and Miller (1982) pointed towards this possibility. By examining humoral antibody responses, they showed that cyclophosphamide pretreatment in BDF_1 mice did not lead to abrogation of oral tolerance but rather to an increase in suppression of antibody responses.

Since the only obvious difference in these two experimental systems was the strain of mice used, I directly compared the effects of cyclophosphamide in these two strains. In repeated experiments, it was

shown that tolerance for CMI was reversed in cyclophosphamide treated animals which had been fed ovalbumin (Figure 4.11). Humoral antibody responses in BDF_1 animals however were lower after cyclophosphamide treatment, whereas BALB/c mice demonstrated the abrogation of systemic tolerance as described before. The reason for this consistent disparity in susceptibility to cyclophosphamide in these two strains of mice is not clear. One possible explanation is a genetic difference in cyclophosphamide induced immune suppression and evidence in support of this possibility has been published by Hurme, Bang & Sihvola (1980).

Effects of cyclophosphamide on systemic immunity

In studying the effects of cyclophosphamide prior to immunisation (Figures 4.12 and 4.13) it was shown that this treatment did not significantly influence either antibody or CMI responses and that it affected both strains in the same way. Thus, the different immunomodulation observed in BALB/c and BDF_1 mice cannot be attributed per se to a general effect of cyclophosphamide on the immune responsiveness.

Modulation of oral tolerance by graft-versus-host response

Small intestinal damage is one of the major pathological consequences of a GvHR in experimental animals and man (Reilly & Kirsner 1965, Slavin & Santos 1973). Mucosal injury may be caused by direct attack of cytotoxic cells (Singh et al 1972) or by release of enteropathogenic lymphokines (Mowat & Ferguson 1981) produced by activated T-cells.

An early sign of a local CMI is an increase in intraepithelial lymphocytes (McDonald & Ferguson 1976,1977, Guy-Grand et al 1978). These intraepithelial cells have been shown to be a heterogeneous population. It consists probably of cytotoxic T-cells (Arnaud-Battandier et al 1978, Davies & Parrott 1981) NK-cells (Parrott et al 1983) and of cells which carry conventional T-helper and T-suppressor lymphocyte markers (Selby et al 1981, Lyscom & Brueton 1982). It is conceivable that these intraepithelial cells, because of their proximity to the gut associated lymphoid tissues and specialised antigen presenting structures may also be responsible for modulating the immune response to fed antigens.

The results demonstrated (Chapter 4) reveal that induction of a GvHR in adult non-irradiated mice led to a signifant increase of the Spleen Index and of intraepithelial lymphocytes without affecting mucosal morphology. In these animals tolerance to ovalbumin was partially reversed for both limbs of the immune response (Figure 4.14). In animals undergoing a GvHR, the immune responsiveness after immunisation with ovalbumin in adjuvant was enhanced, complementing the findings of Treiber & Lapp (1976). Examining the immune responses after injection of sheep erythrocytes during a GvHR, they suggest that the immunological enhancement may be caused by an adherent cell dependent activation of T-helper/amplifier cells. This activation could have altered the mode of antigen presentation and/or processing within the gut associated lymphoid tissues. Whether intraepithelial lymphocytes play a regulatory role in the immune responses discussed above remains to be established. In view of the recent findings regarding nature and immunological functions of the intraepithelial lymphocyte populations found in rodents (Davies & Parrott 1981, Parrott et al 1983, Lyscom &

Brueton 1983), the exact immunological "raison d'être" of a raised intraepithelial lymphocyte count is still a matter of current debate.

Modulation of oral tolerance by muramyl-dipeptide

Investigating further the role of adherent cell populations on the induction or modulation of oral tolerance to a protein antigen, muramyl-dipeptide was used as an immunomodulatory agent. It has been shown to be the simplest structural unit to replace whole killed bacteria in Freund's complete adjuvant (Chedid et al 1977) and to act mainly on macrophages by liberation of monokines (Pabst & Johnston 1980).

Reversal of tolerance to ovalbumin after administration of muramyl-dipeptide supports the hypothesis that macrophage activation and altered antigen presentation can be responsible for this effect. Since my interest was mainly focused on the immunomodulatory effects of this adjuvant, its effects on intestinal morphology have not been investigated. The rapid clearance of muramyl-dipeptide from the circulation (Lederer 1980) and the simultaneous application of antigen and adjuvant make it highly unlikely that morphological alterations could have caused the observed effects.

Modulation of oral tolerance by colonic administration of antigen

Peyer's patches have generally been regarded as important immunological sites within the gut associated lymphoid tissues in regulating the immune responses to ingested antigens (reviewed by Tomasi 1980, Bienenstock & Befus 1980, Walker 1981). Peyer's patches have been shown to generate T-helper cells for local IgA response and T-suppressor cells for systemic IgG responses after a single feed of

antigen (Richman, Graeff, Yarchoan & Strober 1981). After continuous feeding for three weeks, Ngan & Kind (1978) demonstrated T-suppressor cells for IgG and IgE anti-ovalbumin antibody responses within the specialised lyphoid tissues of the Peyer's patch. By feeding lysed sheep erythrocytes for four to five weeks, it was shown that adoptively transferred Peyer's patch cells suppressed IgA and IgG responses *in vitro* leaving IgM responses rather unaffected (McDonald 1982). The unifying theme of these different reports seems to be that Peyer's patches are important organs in the generation of local and systemic immune responses to orally ingested antigen.

Circumvention of Peyer's patches by excision prior to antigen feeds has not been reported in mice. Operative excision would create great technical difficulties and also cause physiological disturbances of the small bowel, for example, due to scarring and altered lymph flow. Thus I decided to use the rectal/colonic route to avoid these problems. The results presented in Chapter 4 demonstrate that colonic administration of ovalbumin induces a profound state of systemic tolerance similar to the tolerance observed after oral feeding. These findings suggest that organised lymphoid follicles (Peyer's patches) are not necessary for the induction of oral tolerance and indicate that intraluminal digestion in the small intestine is not necessary either. There are however small lymphoid follicles in the colon (Kealey 1976) and the presentation of antigen to this part of the gut associated lymphoid tissue is sufficient to induce "oral tolerance". It is theoretically conceivable that the tolerising effects could have been due to ingestion of ovalbumin contaminated faeces after the enema. There was however only unsignificant leakage and mice were carefully monitored for the first hour and faeces were immediately removed to

avoid any oral contact. Thus it seems unlikely that the reported effects are due to the intake of ovalbumin contaminated faeces.

C. ROLE OF THE GUT IN GENERATING ORAL TOLERANCE

As demonstrated in experiments reported in Chapter 4, oral tolerance was abrogated in mice pretreated with cyclophosphamide. It was postulated that this modulation was related to the effects on T-suppressor cells (Röllinghoff et al 1977). However, since cyclophosphamide also acts on rapidly dividing enterocytes it is equally possible that the modulation of oral tolerance by cyclophosphamide was due to alteration in digestion or processing of protein antigens by the gut. Experiments to transfer tolerance by serum of fed donors were designed to investigate these possibilities.

The transfer of tolerance by serum from protein fed mice has not previously been reported. In mice fed repeated doses of sheep erythrocytes, the tolerogenic factors described in serum, all appear to reflect immune responses of the animal to the fed antigen (André et al 1975, Kagnoff 1978b, Kagnoff 1980).

In the interpretation of their experiments on the induction of tolerance to oxazolone, Asherson, Perera and Thomas (1979) suggested that differences in the quantity of systemically available antigen could account for the contrasting effects of oral and parenteral administration of antigen. However it is unlikely that results in my experiments are due to the amount of ovalbumin absorbed since parenteral administration of over a wide range of antigen doses had no tolerising effect on the immune responses of recipients. It is more likely that the tolerogenic properties of the serum from protein fed mice are related to

physico-chemical alterations of the protein occurring in the intestine. After feeding ovalbumin, large amounts of highly deaggregated antigen are found in the serum (Swarbrick 1979) and deaggregated proteins are known to be extremely effective in inducing both oral and parenteral tolerance (Vives, Parks & Weigle 1980, Parks & Weigle 1980b). It has also been shown, using bovine serum albumin, that in vitro proteolytic digestion produces protein fragments which are tolerogenic in vivo, possibly via suppressor cell activation (Dosa et al 1979, Muckerheide, Pesce & Michael 1981, Ferguson, Peters, Reid, Pesce & Michael 1983). Although I have not yet elucidated the nature of circulating immunoreactive ovalbumin after feeding, the results of the experiments are consistent with the hypothesis that tolerogenic forms of protein are produced in vivo after digestion and absorption of protein antigens in the gut, and that these are important in the induction of tolerance.

A striking finding in these experiments has been that transfer of serum from ovalbumin fed donors into naive recipients had no suppressive effect on subsequent systemic humoral immunity of recipients, but did lead to tolerance for CMI. I have found that these two limbs of the immune response differ in their susceptibility to tolerance induction (Chapter 4) by feeding different doses ovalbumin; a low dose of ovalbumin suppressed CMI but not antibody responses. These experiments confirm that there are likely to be more than one regulatory factor involved in modulation of systemic immunity after feeding protein antigens. The tolerance for systemic CMI after feeding ovalbumin was abrogated by cyclophosphamide so was the tolerance for CMI which was observed in recipients of serum from ovalbumin fed mice. The unequivocal observation that cyclophosphamide pretreatment of donors did

not alter the tolerogenic properties of serum obtained after feeding is strong evidence to support the hypothesis that the effects of cyclophosphamide on immune responses to fed ovalbumin are not due to associated changes in the intestinal mucosa but that the induction of oral tolerance for CMI in mice fed ovalbumin is due to a population of cyclophosphamide-sensitive suppressor T-cells which are activated by protein moieties generated within the mucosa of the gastrointestinal tract. In the intact animal, dietary antigens will presumably interact primarily with the suppressor T-cells which other workers have identified within the gut associated lymphoid tissue (Mattingly & Waksman 1978, Richman et al 1978). Parenteral administration of intestinally processed protein produced a state of tolerance for CMI which was identical to the suppression observed after enteral encounter. For this response, it seems to be not essential for the antigen to meet the regulatory cells of the gut associated lymphoid tissues via the intestinal epithelium. The latter route of antigen access and availability within these structures may be of importance for suppression of systemic humoral response. The results underline the close association of absorptive and immunological functions of the intestinal tract, in regulating immune responses to dietary proteins.

Transfer of tolerance by serum of irradiated donors

In a further attempt to investigate the role of the gut in generating systemic hyporesponsiveness, lethal (1000 rad) whole body irradiation was used as a tool to alter the mucosal integrity and immunological responsiveness by modulating the antigen processing and handling abilities of the gut.

The histological changes after irradiation are shown in Figures 5.8 and 5.9 and show profound crypt hyperplasia, villus atrophy and loss of intraepithelial lymphocytes confirming earlier reports (Quastler 1962).

An unexpected finding was the abrogation of transferable tolerance for CMI by irradiation of the serum donors. These findings are novel and experiments are under way to investigate the effect of immediate spleen cell reconstitution on transferable tolerance in this system. Hanson and Morimoto (1982, personal communication) were unable in a different experimental system, however, to induce tolerance in lethally irradiated BDF_1 animals which were reconstituted and fed within two days after reconstitution. They hypothesise that this might have been due to insufficient interaction between transferred cells and absorbed ovalbumin.

In the adoptive transfer system I used, absence or reduced availability of tolerogens could be due to the effects of irradiation on the antigen processing capacity of the gut. Although Bennet, Chastani, Decker & Mead (1951), using radioiodinated human serum albumin, demonstrated that whole body irradiation (600 rad) of mice did not change the rate of protein absorption from the small intestine, I could demonstrate the circulating amounts of antigen in the serum of irradiated animals were higher than the levels detected in non-irradiated controls. It seems that the overall availability of antigen is not the key factor in transferring tolerance for CMI but rather the balance of circulating tolerogens and immunogens. Again, as in the serum transfer experiments, humoral antibody responses were not suppressed but rather primed stressing the fact that CMI and humoral antibody responses are under different immunoregulatory control.

Further experiments investigating the the effects of irradiation with or without reconstitution prior to feeding are necessary to elucidate the intriguing underlying mechanisms which tilt the immunoregulatory balance from tolerance to sensitisation and to answer the question why antibody responses are not suppressed by this treatment.

D. EFFECTS OF AGE ON INDUCTION OF ORAL TOLERANCE

Feeding of a weight related dose of ovalbumin to adult mice consistently produces tolerance. I have, however, demonstrated that when an equivalent dose is fed to neonatal mice within the first week of life, oral tolerance does not occur (Chapter 6). Furthermore, animals fed on day one of life were primed, and when foetuses were exposed to antigen via intraamniotic administration, an even greater priming of subsequent systemic immune responses occurred. These results confirm and extend one previous report (Hanson 1981) that a single feed of ovalbumin to mice on the first day of life primes for subsequent antibody responses. I attempted to modulate this priming phenomenon in two ways - by a retolerisation feed and by varying the antigen dose and frequency of administration. The priming effects persisted for several weeks but it proved possible to create a state of tolerance for systemic antibody responses by another tolerising feed four weeks after the neonatal feed; for CMI, however, the state of non-tolerance persisted for 14 weeks (Figure 6.10). In addition, I demonstrated that several daily doses of antigen (starting on day one) induced tolerance whereas the same total dose fed on a single occasion did not (Table 6.9).

Failure to induce tolerance by an important physiological route of antigen exposure is in striking contrast to the generally recognised pattern that neonatal mice are very readily tolerised by antigen given by other routes (Billingham et al 1953, Chiller & Weigle 1971, Titus et al 1981). It has been reported that a feed of human gamma globulin tolerises neonatal mice, which contrasts the priming effect observed after feeding ovalbumin (Hanson 1981) and this was interpreted to be a dose related phenomen. Human gamma globulin is however an atypical substance to use as an antigen for investigating immune responses to dietary antigens, in view of its preferential absorption and handling by the neonatal gut (Brambell 1966, Morris 1968). The development of the small intestine in baby mice occurs in stages with a rapid series of changes around the time of weaning in the third week of life. The immunological effects of fed antigen also follows an age related pattern with the change from non-tolerance to tolerance at about 10 days. This is before closure (Morris 1968, Walker 1979), brush border enzyme development (Ferguson, Gerskovitch & Russell 1973), intraepithelial lymphocyte and plasma cell infiltration of villi (Ferguson & Parrott 1972). On the other hand, the organised lymphoepithelial organs including Peyer's patches are already populated by T-cells three to four days after birth (Joel, Hess & Cottier 1971, 1972). Thus there are no clear connections between changes in <u>in vivo</u> immune effects and the well established patterns of enterocyte and lymphoid maturation of the gut. The general pattern of increasing ease of tolerance induction after 10 days of age was briefly interrupted around the time of weaning. This was related to the weaning and feeding interval and not to age. At weaning there is not only exposure to many new dietary antigens, but

also withdrawal of maternal milk and changes in gut flora. All of these are candidate mechanisms for the temporary reduction of tolerance I observed. The factors responsible for neonatal priming may be the same as those operative around weaning, or there may be entirely different mechanisms involved in the specific perinatal patterns of immune responsiveness of mice.

Experiments in adult mice have shown that several different immunological mechanisms are involved in oral tolerance as discussed earlier. Immaturity of any of these immunological functions could explain the absence of oral tolerance, and the priming effect produced by feeding neonates and foetuses. Nevertheless, it is known that the foetus responds to antigenic stimuli, can be immunised prior to birth (Cramer, Kunz & Gill 1974), and that foetal or neonatal cells can act as adult cells when they are transferred into an adult environment (Dixon & Weigle 1959). It has been established that there exists in neonatal, but not in adult mice, a population of nonspecific suppressor cells (review: Murgita & Wigzell 1981; Stephenson, Dorsch, Roser, Godden & Herbert 1983). It seems unlikely that these have a role in perinatal priming but this is still conceivable in view of the complex T-T cell interactions involved in an orderly immune response. Hanson (1981) produced evidence that priming for antibody responses in the neonate is due to direct effects of antigen on B-cells. However since I have shown effects on systemic CMI too, there must also be direct or indirect T-cell involvement.

The immunological effects of feeding in the neonatal period may, however, be unrelated to the state of maturity of the lymphoid system. It is equally possible that immaturity of the neonatal digestive and

absorptive functions is the key factor (Clark 1959). The permeability of the neonatal gut to macromolecules is increased, as, for example, demonstrated by high concentrations of serum antigen after feeding (Warshaw, Walker & Isselbacher 1974, Eastham, Lichauco, Grady & Walker 1978). Although the quantity of a circulating antigen may be important, immunochemical properties and characteristics of absorbed antigen may be equally relevant. Deficiency in the digestive functions of the gut may yield antigenic moieties which are more immunogenic than normal, instead of generating tolerogenic fragments (see above). It has also been shown that in vitro digestion of bovine serum albumin leads to generation of both tolerogenic and immunogenic fragments (Dosa et al 1979), and so it is conceivable that disturbances in the digestion of ovalbumin are an explanation for the unusual immune responses observed after feeding this antigen to the newborn rodent.

A working hypothesis to explain the findings of these series of experiments has to take into account digestive and immunological functions, and the fact that there are differences in the pattern of retolerisation for antibody and CMI responses. Based on published reports and my own experiments, I suggest that an area of intestine generates antigenic moieties which stimulate a T-inducer/helper cell population rather than T-suppressor cells. The differences between antibody and CMI responses can be explained if memory cells for antibody responses have a shorter lifespan than T-effector or memory cells for delayed type hypersensitivity responses. In support of this hypothesis, it has been shown, although in a different experimental system, that T_{DTH} effector cells may be long lived (Shiho, Takaoki, Nakagawa, Arakawa & Takeuchi 1982). Much further work will be required to

investigate the role of the gut as a regulator of immune responses to fed antigens and to examine the subsequent systemic immune responses at the cellular level. This should be directed towards the documentation of development of the mucosal immune system, both with respect to immunoregulatory and effector functions. It will also be essential to examine the immunochemical properties of immunogenic materials which are found in blood after enteral administration. My experiments have addressed only humoral antibody responses of the IgG and IgM class (by passive haemagglutination and ELISA techniques) and CMI responses by measuring delayed type hypersensitivity with a skin test. Subsequent work should also concern the effects of neonatal antigen exposure on IgE and mucosal IgA antibody responses.

E. INDUCTION OF MUCOSAL CELL MEDIATED IMMUNITY

Mucosal CMI is a common feature in GvHD and the intestine is a primary target organ (Slavin & Santos 1973). Using morphometry and stathmokinetic techniques (Mowat & Ferguson 1981,1982), it was demonstrated that induction of GvHR in one week old neonatal mice led to significant changes in intraepithelial lymphocytes and infiltration of mast cells in the lamina propria. In addition, they observed an increase in crypt cell production rates and in crypt lengths. Induction of GvHR in adult mice led to similar changes of intestinal morphology. The general health of these animals was unaffected and all groups showed a normal weight gain (Mowat 1981). If, however, as in my experiments, a GvHR was induced in one day old animals, they developed overt GvHD with diarrhoea, wasting and death occuring between the 10th and 14th day after injection of parental spleen cells. This

was mirrored by increases in intraepithelial lymphocytes, increases in crypt lengths, shortening of villi and a concomitant splenomegaly. Shortening of villi is considered a late event in the intestinal epithelial damage (Mowat & Ferguson 1982), presumably being the consequence of a maximally stimulated cell turnover ("hyperplastic villus atrophy").

My results confirm the role of the gut as a target organ in neonatal GvHD and prove the validity of the techniques used to assess local intestinal CMI.

Nature of cellular infiltration during a local cell mediated immune reaction: intraepithelial lymphocytes

The T-cell lineage of intraepithelial lymphocytes has been demonstrated by various groups (Ferguson & Parrott 1972, Guy-Grand et al 1974,1978, Selby et al 1981), their exact role and nature however remain unresolved to date.

In humans, the majority of intraepithelial lymphocytes are reported to bear the suppressor phenotypic marker (Janossy et al 1980) which has also been demonstrated for rats (Lyscom & Brueton 1982) whereas the T-helper phenotype is frequently found in the lamina propria. There is further more - still conflicting - evidence of natural killer cells and cytotoxic activity detectable in small intestinal intraepithelial lymphocytes. In guinea pigs, both natural killer and killer cell activities are detectable among intraepithelial lymphocytes (Arnaud-Battandier et al 1978). In mice, cytotoxic T-cell activity (Davies & Parrott 1981) and natural killer cell activity has been shown in analysing almost pure intraepithelial lymphocyte preparations (Parrott

et al 1983). These reports and studies identifying human natural killer effector cells as granulated lymphocytes (Timonen, Ortaldo & Herberman 1981) support the concept that the intraepithelial lymphocytes are a heterogeneous population of cells. It seems feasible that these cells play an important role in mucosal defence and immune surveillance.

Further studies using monoclonal antibodies against cell surface markers as well as functional studies are needed to elucidate which population of intraepithelial cells may be responsible for the rise in numbers during a local intestinal CMI.

Mucosal mast cells

The origin of mucosal mast cells is still a matter of conflicting reports and a T-cell origin (Guy-Grand et al 1978) of mucosal mast cells and a bone marrow origin of connective tissue type mast cells have been proposed (Kitamura et al 1977,1981). Recent studies support the latter findings and suggest that mucosal mast cell precursors are also of bone marrow origin (Haig, MacKee, Jarrett, Woodbury & Miller 1982). It is now becoming obvious that neither population of mast cells is derived directly from T-lymphocytes (Mayrhofer & Bazin 1981) but that mucosal mast cells require under certain conditions, for example, during parasite infection (Ruitenberg & Elgersma 1976) an inductive influence provided by T-lymphocytes. Mucosal mast cells are involved in delayed type hypersensitivity responses (Askenase 1980) and have been shown to be part of the cellular infiltration during a GvHR (Guy-Grand et al 1978, Mowat & Ferguson 1982).

Mucosal mast cell counts and counts of granulated intraepithelial lymphocytes were performed in the experiments presented in this thesis

to find out whether an increase of granulated cells within the lamina propria and epithelium would be an essential part of a local intestinal CMI. Preservation of mucosal mast cells is dependent on the appropriate fixative (Enerbäck 1966a) and the results presented in this thesis were obtained by using Carnoy's fluid, a fixative which is known to preserve mucosal mast cells.

In my studies I did not observe a significant change in mucosal mast cell infiltration during a local CMI. This finding, however, does not imply that these cells do not play a role in CMI responses. Mucosal mast cells can discharge their mediators without total disruption of their cell membrane and could thus be still recognisable as intact cells under the light microscope.

A striking and unexpected finding was that nearly 100% of intraepithelial lymphocytes of the (CBA x BALB/c)F_1 hybrid generation were granulated. This is in sharp contrast to the numbers found in both parental strains, where granulated cells contributed to less than 1% of the total intraepithelial lymphocyte population. Parrott et al (1983) reported that up to 60% of intraepithelial lymphocytes are granulated and stain positively with astra blue or Giemsa stain.

Further experiments are needed to clarify the exact nature of the granulated cell populations and to examine the mode of inheritance and whether, for example, a high number of granulated intraepithelial lymphocytes may represent a selective advantage.

Abrogation of oral tolerance and induction of local mucosal cell mediated immunity: cyclophosphamide

Feeding ovalbumin to adult rodents leads to systemic tolerance. Systemic immunisation, however, has also been shown to occur after oral

antigen administration (Perrotto et al 1974). A local intestinal CMI has been shown in pigs (Huntley, Newby & Bourne 1979) after feeding sheep erythrocytes. In these reports the CMI was only short lived and disappeared within one week. In a different system, intestinal CMI has been demonstrated in mice after the immune responsiveness has been modulated by cyclophosphamide prior to feeding (Mowat & Ferguson 1981). As I have shown earlier, cyclophosphamide also abrogated the induction of oral tolerance and these results were the rationale for investigating whether the abrogation of oral tolerance would lead to a local CMI on re-exposure to the same antigen.

In repeating published work (Mowat & Ferguson 1981), I confirmed that immunomodulation with cyclophosphamide abrogates oral tolerance and does lead to an infiltration of the mucosa with intraepithelial lymphocytes, whereas oral immunisation without cyclophosphamide treatment was ineffective. Crypt changes and changes in the crypt cell production rates however were variable and never reached significant levels. The reasons for this discrepancy remain unclear; among the candidates mechanisms responsible for this difference could be a change in the intestinal flora of these animals and in this way altering the adjuvanticity of the bacterial gut flora (Wannemuehler et al 1981). It is also conceivable that the duration of the antigen challenge may have been inappriopriately long and has rather led to induction of tolerance (see Chapter 6).

To investigate this possibility, single feeds rather than continuous challenges have been examined. A single challenge of cyclophosphamide pretreated animals with 25 mg or as little as 100 µg ovalbumin produced a significant rise in intraepithelial lymphocytes

after only 24 hours. This demonstrates the sensitivity of the intraepithelial lymphocytes response on one hand and that their numbers, on the other hand, stay on an elevated level during the period of challenge. Although I investigated only two time-points, the first day and last day of the challenge period, it is unlikely that the intraepithelial lymphocyte infiltration would fluctuate during the time of continuous antigen presentation. Based on the results of these experiments it is obvious that feeding for 10 days does not induce oral tolerance in previously sensitised adult animals. Taking published reports (McDonald & Ferguson 1976, Guy-Grand et al 1978, Mowat 1981, Mowat & Ferguson 1982) and here presented evidence together, it is obvious that the intraepithelial lymphocyte response within the gut is a reliable marker of a mucosal CMI response. The observations discussed here demonstrate that an increase in intraepithelial lymphocytes can be the only sign of a local CMI; an elevated crypt cell production rate and morphological changes are not inevitably consequences of a local intestinal CMI.

Graft-versus-host reaction

Induction of GvHR leads to an intestinal CMI and mice being fed ovalbumin during the proliferative phase of a GvHR failed to develop systemic hyporesponsiveness. In an attempt to recreate villus and crypt abnormalities similar to those observed in human food related diseases (for example, coeliac disease, cows' milk protein intolerance), I tried to augment the intestinal changes by inducing an additional delayed hypersensitivity reaction to a different antigen. It was assumed that abrogation of oral tolerance, at the same time, would prime the animals for intestinal delayed hypersensitivity reactions as seen after treatment with cyclophosphamide and muramyl-dipeptide.

Following the general protocol for the induction of mucosal CMI, a similar infiltration with intraepithelial lymphocytes was observed in both experimental groups without other morphological changes (Figure 7.11). The failure to demonstrate augmented changes due to an additional CMI response to ovalbumin proved this hypothesis to be incorrect. The possibility, however, that the assay system was not sensitive enough to measure additional effects cannot be ruled out. Another way to explain these findings would be that oral tolerance is maintained by different immunoregulatory mechanisms and modulation or abrogation of only one part of this network (systemic tolerance) does not necessarily allow a local cell mediated immune response to take place.

Muramyl-dipeptide

Like other adjuvants, injection of muramyl-dipeptide can lead to immunostimulation or immunosuppression depending on the time of application in relation to the antigen (Leclerc et al 1979).

In the experiments described to examine the effects of muramyl-dipeptide on the systemic immune responses after oral antigen administration, it was given concomitantly but via different routes. Having demonstrated the abrogation of systemic tolerance to ovalbumin by this treatment, I decided then to find out whether the gut associated lymphoid tissues may have been primed for cell mediated immunity by this procedure. Figure 7.13 clearly shows that this was the case and the combination of muramyl-dipeptide and ovalbumin induced a local cell mediated immune reponse when the animals were challenged with the antigen for 10 days. Muramyl-dipeptide has complex effects on the immune system and has been shown to enhance antibody dependent cell

mediated cytotoxicity by activation of macrophages (Löwy et al 1977, Fevrier et al 1978). Furthermore, induction of T-helper cells has been demonstrated and with it the need of T-cell co-operation for muramyl-dipeptide to exert its effects on the immune system (Löwy et al 1977). T-helper cells have also been postulated to be more readily activated (Morris & Johnston 1978). The observation that muramyl-dipeptide injected, ovalbumin fed and immunised mice responded with higher antibody titres compared to their saline fed controls (Hanson 1982, personal communication) tends to support the hypothesis that antigen specific T-helper cells may have been primed.

Following these lines of evidence, it is conceivable that the net gain in T-help (through macrophage activation or through an increase in T-help for other reasons) leads to the sensitisation of the gut associated lymphoid tissues and finally to intestinal CMI. This interpretation, however, is still speculative and cell culture experiments with identification of functional T-cell subsets and cell transfer experiments should be performed in future to answer these important questions.

The effects of age at first feed on induction of local CMI

Priming of the systemic immune system observed in mice which had been fed ovalbumin on the first day of life was a complete novel finding and the possible underlying mechanisms have been discussed earlier. These findings prompted further investigations to find out whether the systemic effects could be identified at the mucosal level too.

I demonstrated in repeated experiments that BALB/c mice fed ovalbumin on the first day of life responded to a later challenge with a significant rise in intraepithelial lymphocytes. This is suggestive of a

local CMI, although no other morphological changes were observed. It is not clear why CBA mice treated and tested in the same way developed intestinal mucosal changes (Figure 7.14) but no rise in intraepithelial lymphocytes. Both strains of mice differ genetically in the major histocompatibility complex and presumably in their immune responsiveness to ovalbumin (Levine & Vaz 1970, Swarbrick 1979, Stokes et al 1983). Furthermore, there is some speculative evidence that differences in the activity of a regulatory contrasuppressor circuit to sheep erythrocytes within the Peyer's patch (Green, Gold, St Martin, Gershon & Gershon 1982) may be closely linked to specific haplotypes of the major histocompatibility complex and could account for a different immunoregulation within the micro-environment of the Peyer's patch. Much further work however is needed to explore these findings in depth and to substantiate this attractive hypothesis of Green et al (1982).

It seems that a net gain in T-help has been induced by feeding ovalbumin to neonatal mice. This immune response overrides the usually observed dampening of the immune responsiveness of the gut associated lymphoid tissues by "traditional" T-suppressor cells (Richman et al 1981) and/or by induction of an inhibitory feedback pathway by Lyt 1+ suppressor inducer cells in the Peyer's patch (McDonald 1982).

F. CONCLUSION AND CLINICAL SIGNIFICANCE

Immune responses to fed antigen are extremely complex and enteral exposure to antigens may produce systemic tolerance or less freqently immunisation and/or local immunity in the intestinal mucosa. After an antigen feed, various responses at the cellular level are responsible for the induction and maintenance of oral tolerance which involves T-

helper, T-suppressor cells, B-cells, macrophages and several, still hypothetical and possibly complementary immunoregulatory circuits (Richman et al 1981, Green et al 1982, McDonald 1982). Modulation of these responses by cyclophosphamide, muramyl-dipeptide and a GvHR leads to abrogation of oral tolerance by elimination of T-suppressor cells and presumably by activation of macrophages and/or a T-helper inducer cell circuit.

Immunosuppressive or immunoenhancement therapy is widely used in treatment of malignant disease and since gastrointestinal side effects are frequently encountered, it will be important to investigate whether these are consequences of direct mucosal cell injury or due to the breakdown of the homeostatic mechanisms controlling oral tolerance. The quantity of antigenic material taken up by the intestine has been proposed to be an important regulator of intestinal immune responses (Walker 1981). However, abrogation of transferable tolerance after irradiation of serum donors and concomitant demonstration of an increased quantity of circulating ovalbumin would suggest a different explanation. It is not merely the quantity but the immunological quality of absorbed antigen which regulates the ensuing immune response. Demonstration of priming of the immune responses by feeding neonatal mice would point in a similar direction. In the context of these studies, observation of an even more pronounced priming effect after intraamniotic antigen exposure ("feeding") suggest a crucial role of the gut in regulating immune responses to fed antigen.

Several diseases of infancy which cause enteropathy and malabsorption are closely associated with hypersensitivity to food antigens. Although cows' milk protein intolerance and persistent

intolerance to wheat (coeliac disease) are frequently found in infancy, theoretically each antigen introduced during a vulnerable period can cause a malabsorption syndrome and recent reports confirm this theory (Vitoria, Camarero, Sajo, Ruiz & Rodriguez-Soriano 1982). Intestinal lesions consist of crypt hyperplasia, villus atrophy and lymphocytic infiltration of the epithelium (Kuitunen et al 1974, Mavromichalis et al 1976, Ferguson 1977, Phillips et al 1979) and it seems likely that those changes reflect an intestinal CMI response to dietary antigens.

The work described in this thesis indicates that oral tolerance is induced and maintained by a complex immunoregulatory circuit including T-suppressor, T-helper/inducer cells and that antigen presenting cells play an important role in these circuits. Feeding ovalbumin to mice during the perinatal period and after immunomodulation by drugs abrogates the protective effects of oral tolerance and induces a local intestinal CMI response on antigen re-exposure in later life.

These findings bear obvious resemblances to clinical observations made in gastroentropathies which have been caused, for example, by cows' milk protein intolerance. In these cases, a single postnatal feed of cows' milk to breast fed babies is a sufficient antigenic stimulus to lead to early sensitisation and priming for immediate and delayed type hypersensitivity responses in later life.

The experimental work described within this thesis provides a structured basis for investigation of an important clinical problem: that feeding of protein antigens to human neonates - at a time when both digestive and immune systems are immature - sometimes sensitises the infant for potentially harmful immune responses to foods, and thereby to food allergic diseases.

REFERENCES

Affinity chromatography. Principles and methods.
Pharmacia Fine Chemicals AB, Uppsala, Sweden 1979

Aldo-Benson, M., Borel, Y. The tolerant cell. Direct evidence for receptor blockade by tolerogen.
J.Immunol. 1974; 112: 1793-1803

Anderson, R.E., Sprent, J., Miller, J.F.A.P.
Radiosensitivity of T and B lymphocytes. I. Effect of irradiation on cell migration.
Eur. J. Immunol. 1974; 4: 199-203

Anderson, R.E., Warner, N.L.
Radiosensitivity of T and B lymphocytes. III. Effect of irradiation on immunoglobulin production by B-cells.
J. Immunol. 1975; 115: 161-169

André, C., Heremans, J.F., Vaerman, J.P., Cambiaso, C.L.
A mechanism for the induction of immunological tolerance by antigen feeding: antigen - antibody complexes.
J. Exp. Med. 1975; 142: 1509-1519

Andrew, E., Hall, J.G.
IgA antibodies in the bile of rats. II. Evidence for immunological memory in secretory immunity.
Immunology 1982; 45: 176-182

Arnaud-Battandier, F., Bundy, B.M., O'Neill, M., Bienenstock, J. Nelson, D.L.
Cytotoxic activities of gut mucosal lymphoid cells in guinea pigs.
J. Immunol. 1978; 121: 1059-1065

Arnaud-Battandier, F., Wahl, S.M., Nelson, D.L. Functional properties of intestinal mucosal lymphoid cells from guinea pigs. Fed. Proc. 1979; 38: 1017

Asherson, G.L., Zembala, M., Perera, M.A.C.C., Mayhew, B., Thomas, W.R. Production of immunity and unresponsiveness in the mouse by feeding contact sensitising agents, and the role of suppressor cells in the Peyer's patches, mesenteric lymph nodes and other lymphoid tissues.
Cell. Immunol. 1977; 33: 145-155

Asherson, G.L., Perera, M.A., Thomas W.R.
Contact sensitivity and the DNA response in mice to high and low doses of oxazalone, low dose unresponsiveness following painting and feeding and its prevention by pretreatment with cyclophosphamide.
Immunology 1979; 36: 449-459

Asherson, G.L, Zembala, M.
T suppressor cells and suppressor factor which act at the efferent stage of the contact sensitivity skin reaction: their production by mice injected with water soluble, chemically reactive derivatives of oxazolone and picrylchloride.
Immunology 1980;42: 1005-13

Askenase, P.W., Hayden, B.J., Gershon, R.K.
Augmentation of delayed type hypersensitivity by doses of cyclophosphamide which do not affect antibody responses.
J. Exp. Med. 1975; 141: 697-702

Askenase, P.W.
Immunopathology of parasitic diseases: involvement of basophils and mast cells.
Springer Sem. Immunopathol. 1980; 2: 417-442

Basten, A., Miller, J.F.A.P., Sprent, J., Cheers, C.
Cell to cell interaction in the immune reaction. X.T-cell dependent suppression in tolerant mice.
J. Exp. Med. 1974; 140: 199-217

Bennett, L, Chastain, S., Decker, A., Mead, J.
Effect of roentgen irradiation upon protein absorption in the mouse.
Proc. Soc. Exp. Biol. Med. 1951; 77: 715-718

Bienenstock, J., Befus, A.D.
Mucosal immunology.
Immunology 1980; 41: 249-270

Billingham, R.E., Brent, L., Medawar, P.B.
Actively acquired tolerance of foreign cells.
Nature 1953; 172: 603-606

Billingham, R.E., Brent, L.
A simple method for inducing tolerance of skin homografts in mice.
Transplant. Bulletin 1957; 4: 67-71

Bloom, G., Kelly, J.W.
The copper pthalocyanin dye 'Astrablau' and its staining properties, especially the staining of mast cells.
Histochemie 1960; 2: 48-57

Brambell, F.W.
The transmission of immunity from mother to young and the catabolism of immunoglobulins.
Lancet 1966; 2: 1087-1093

Bretscher, P.A., Cohn, M.
A theory of self-nonself discrimination.
Science 1970; 169: 1042-1049

Burnet, F.M., Fenner, F., eds.
The production of antibodies. 2nd ed., Melbourne London: Macmillan 1949

Burnet, F.M., ed.
The clonal selection theory of acquired immunity.
Cambridge New York: Cambridge University Press: 1959

Burnet, F.M.
The probable relationship of some or all mast cells to the T-cell system.
Cell. Immunol. 1977; 30: 358-360

Cantor, H., Boyse, E.A.
Functional subclasses of T-lymphocytes bearing different Ly antigens. I The generation of functionally distinct T-cell subclasses in a differentiative process independent of antigen.
J. Exp. Med. 1975; 141: 1376-1389

Cantor, H., Boyse, E.A.
Regulation of the immune response by T-cell subclasses.
Contemp. Top. Immunobiol.1977; 7: 47-67

Cantor, H.M., Dumont, A.E.
Hepatic suppression of sensitization to antigen absorbed into the portal system.
Nature 1967; 215: 744-745

Chakravarty, A.K.
An in vitro study of functional maturation of murine thymus cells.
Differentiation 1977; 8: 21-29

Challacombe, S.J., Tomasi, T.B.
Systemic tolerance and secretory immunity after oral immunisation.
J. Exp. Med. 1980, 152: 1459-1472

Chalon, M.P., Milne, R.W., Vaerman, J.P.
In vitro immunosuppressive effect of serum from orally immunized mice.
Eur. J. Immunol. 1979; 9: 747-751

Chase, M.W.
Inhibition of experimental drug allergy by prior feeding of the sensitising agent.
Proc. Soc. Exp. Biol. Med. 1946; 61: 257-259

Chedid, L., Parant, M., Parant, F., Lefrancier, P., Choay, J. Lederer, E.
Enhancement of nonspecific immunity to Klebsiella pneumonia infection by a synthetic immunoadjuvant (N-acetylmuramyl-L-alanyl-D-isoglutamine) and several analogs.
Proc. Natl. Acad. Sci. USA 1977; 74: 2089-2093

Chiller, J.M., Habicht, G.S., Weigle, W.O.
Kinetic differences in unresponsiveness of thymus and bone marrow cells.
Science 1971; 171:813-815

Chiller, J., Weigle, W.O.
Cellular events during induction of immunologic unresponsiveness in adult mice.
J. Exp. Med. 1971; 106: 1647-1653

Chiller, J.M. Titus, R.G., Etlinger, H.M.
Cellular dissection of tolerant states induced by the oral route or in neonatal animals.
In: Baram, P., Battisto, J.R., Pierce, N.F., eds., Immunologic tolerance and macrophage function. Elsevier North-Holland, Developments in Immunology 1979: 4: 195-221

Claman, H.N., Phanuphak, P., Moorhead, J.W.
Tolerance to contact sensitivity - A role for supressor T-cells?
In: Katz, D.H., Benacerraf, B., eds., Immunological tolerance
New York: Academic Press 1974: 123-128

Claman, H.N., Miller, S.O., Moorhead, J.W.
Tolerance: two pathways of negative immunoregulation in contact sensitivity to DNFB.
Cold Spring Harbor Symp. Quant. Biol. 1977; 41: 105-111

Claman, H.N.
T-cell tolerance - one signal?
Cell. Immunol. 1979; 48: 201-207

Clark, S.L.
The ingestion of proteins and colloidal materials by columnar absorptive cells of the small intestine in suckling rats and mice.
J. Biophys. Biochem. Cytol. 1959; 5: 41-50

Clarke, R.M.
Mucosal architecture and epithelial cell production rate in the small intestine of the albino rat.
J. Anat. 1970; 107: 519-529

Cooper, M.G., Ada, G.L.
Delayed type hypersensitivity in the mouse. III. Inactivation of thymus derived effector cells and their precursors.
Scand. J. Immunol. 1972; 1: 247-253

Cramer, D.V., Kunz, H.W., Gill, T.J.
Immunologic sensitization prior to birth.
Am. J. Obstet. Gynecol. 1974; 120: 431-439

Dakin, R.
Remarks on a cutaneous affection produced by certain poisonous vegetables.
Am. J. Med. Sci. 1829; 4: 98-100

Davies, M.D., Parrott, D.M.V.
Cytotoxic T-cells in small intestinal epithelial, lamina propria and lung lymphocytes.
Immunology 1981; 44: 367-371

Davis, W.E., Cole, L.J., Schaffer, W.T.
Graft-versus-host reactions in nonirradiated mice. Early suppression of Jerne plaques and hemopoietic colony-forming units.
Transplantation 1970; 9: 529-537

Detrick, L.E., Latta, H., Upham, H.C., McCandless, R.
Electron-microscopic changes across irradiated rat intestinal villi.
Radiat. Res. 1963; 19: 447-461

Diener, E., Feldman, M.
Relationship between antigen and antibody induced suppression of immunity.
Transplant.Rev. 1972; 8: 76-103

Dixon, F.J., Weigle, W.O.
The nature of immunological inadequacy of neonatal rabbits.
II. Antibody formation by neonatal spleenic cells transferred to adult recipients.
J. Exp. Med. 1959; 110: 139-146

Dosa, S., Pesce, A.J., Ford, D.J., Muckerheide, A., Michael, J.G.
Immunological properties of peptic fragments of bovine serum albumin.
Immunology 1979; 38: 509-517

Dresser, D.W.
Specific inhibition of antibody production. II.Paralysis induced in adult mice by small quantities of protein antigen.
Immunology 1962; 5: 378-388

Dresser, D.W., Mitchison, N.A.
The mechanism of immunological paralysis.
Adv. Immunol. 1968; 8: 129-181

Dukor, P., Tarcsay, L., Baschang, G.
Immunostimulants.
Ann. Rep. Med. Chem. 1979; 14: 146-167

Eardley, D.D., Hugenberger, J., McVay-Boudreau, L., Shen, F.W., Gershon, R.K., Cantor, H.
Immunoregulatory circuits among T-cell sets. I.T-helper cells induce other T-cell sets to exert feedback inhibition.
J. Exp. Med. 1978; 147: 1106-1115

Eastham, E.J., Lichauco, T., Grady, M.I., Walker, W.A.
Antigenicity of infant formulas: role of immature intestine in protein permeability.
J. Pediatr. 1978; 93: 561-564

Ecknauer, R., Löhrs, V.
The effect of a single dose of cyclophosphamide on the jejunum of specified pathogen free and germ free rats.
Digestion 1976; 14: 269-280

Ehrlich, P.
Gesammelte Arbeiten zur Immunitätsforschung
Hirschwald, Berlin; 1904

Elson, C.O., Reilly, R.W., Rosenberg, I.H.
Small intestinal injury in the GvHR: an innocent bystander phenomenon.
Gastroenterology 1977; 72: 886-889

Elson, C.O., Heck, J.A., Strober, W.
T-cell regulation of murine IgA synthesis.
J. Exp. Med. 1979; 149: 632-643

Endres, R.O. Grey, H.M.
Antigen recognition by T-cells. I.suppressor T-cells fail to recognise cross reactivity between native and denatured ovalbumin.
J. Immunol. 1980a ; 125: 1515-1520

Endres, R.O., Grey, H.M.
Antigen recognition by T-cells. II. Intravenous administration of native or denatured ovalbumin results in tolerance to both forms of antigen.
J. Immunol. 1980b; 125: 1521-1525

Enerbäck, L.
Mast cells in rat gastrointestinal mucosa. I. Effects of fixation.
Acta Path. Microbiol. Scand. 1966a;66: 289-302

Enerbäck, L.
Mast cells in rat gastrointestinal mucosa. II. Dye-binding and metachromatic properties.
Acta Path. Microbiol. Scand. 1966b;66: 303-312

Engvall, E., Pesce, A.J., eds.
Quantitative enzyme immunoassay.
Scand. J. Immunol. 1978; 8 (Suppl. 7): 1-129

Etlinger, H., Chiller, J. .
Maturation of the lymphoid system. I. Induction of tolerance in neonates with a T dependent antigen that is an obligate immunogen in adults.
J. Immunol. 1979; 122: 2558-2563

Feldmann, M.

T-cell suppression in vitro. I. Role in regulation of antibody responses.

Eur. J. Immunol. 1974; 4: 660-666

Feldmann, M., Beverley, P.C., Erb, P., Howie, S., Kontiainen, S., Maoz, A., Mathies, M., McKenzie, I., Woody, J.,

Current concepts of the antibody response: heterogeneity of lymphoid cells interactions and factors.

Cold Spring Harbor Symp. Quant. Biol. 1977; 41 pt. 1:113-118

Ferguson, A., Murray D.

Quantitation of intraepithelial lymphocytes in human jejunum.

Gut 1971; 12: 988-994

Ferguson, A., Parrott, D.M.V.

The effect of antigen deprivation on thymus dependent and thymus independent lymphocytes in the small intestine of the mouse.

Clin. Exp. Immunol. 1972; 12: 477-488

Ferguson, A.

Intestinal immunity. The role of the intraepithelial lymphocyte.

Ph.D. Thesis, University of Glasgow 1973

Ferguson, A., Gerskovitch, V.P., Russell, R.I.

Pre and postweaning disaccharidase patterns in isografts of fetal mouse intestine.

Gastroenterology 1973; 64: 292-297

Ferguson, A., Parrott, D.M.V.

Histopathology and time course of rejection of allografts of mouse small intestine.

Transplantation 1973; 15: 516-554

Ferguson, A.
Lymphocytes in Coeliac disease.
In:Hekkens, W.T.J.M., Pena, A.S. eds., Coeliac disease
London: Stenfert-Kroese 1974: 265-276

Ferguson, A., Jarrett, E.E.E.
Hypersensitivity reactions in the small intestine. I. Thymus dependence of experimental 'partial villus atrophy'.
Gut 1975; 16: 114-117

Ferguson, A.
Intraepithelial lymphocytes of the small intestine.
Gut 1977; 18: 921-937

Ferguson, T.A., Peters, T., Reed, R., Pesce, A.J., Michael, J.G.
Immunoregulatory properties of antigenic fragments from bovine serum albumin.
Cell. Immunol. 1983; 78: 1-12

Fevrier, M., Birrien, J.L., Leclerc, C., Chedid, L., Liacopoulos, P.
The macrophage, target cell of the synthetic adjuvant muramyl dipeptide.
Eur. J. Immunol. 1978; 8: 558-562

Fichtelius, K.E., Yunis, E.J., Good, R.A.
Occurrence of lymphocytes within the gut epithelium of normal and neonatally thymectomised mice.
Proc. Soc. Exp. Biol. Med. 1968; 128: 185-188

Fichtelius, K.E., Finstad, J., Good, R.A.
The phylogenetic occurrence of lymphocytes within the gut epithelium.
Int. Arch. Allergy Appl. Immunol. 1969; 35: 119-133

Fidler, J.M., Chiscon, M.O., Golub, E.S.
Functional development of the interacting cells in the immune response. II. Development of immunocompetence to heterologous erythrocytes in vitro.
J. Immunol. 1972; 109: 136-140

Germain, R.N., Benacerraf, B.
Helper and suppressor T-cell factors: a review.
Springer Sem. Immunopathol. 1980; 3: 93-127

Gershon, R.K., Kondo, K.
Cell interactions in the induction of tolerance. The role of thymic lymphocytes.
Immunology 1970; 18: 723-737

Gershon, R.K., Kondo, K.
Infectious immunological tolerance.
Immunology 1971; 21: 903-914

Gershon, R.K.
A disquisition on suppressor T-cells.
Transplant. Rev. 1975; 26: 170-185

Gill, H.U., Liew, F.Y.
Regulation of delayed-type hypersensitivity. III. Effect of cyclophosphamide on the suppressor cells for delayed-type hypersensitivity to sheep erythrocytes in mice.
Eur. J. Immunol. 1978; 8: 172-176

Glaister, J.R.
Light, fluorescence and electron microscopic studies of lymphoid cells in the small intestinal epithelium of mice.
Int. Arch. Allergy Appl. Immunol. 1973; 45: 854-867

Godfrey, H.P.,
The use of 1-fluoro -2,4 dinitrobenzene as an affinity label for the antigen receptor of delayed hypersensitivity.
Immunology 1976; 31: 665-673

Goidl, E.A., Siskind, G.W.
Ontogeny of B-lymphocyte function. I. Restricted heterogeneity of the antibody response of B-lymphocytes from neonatal and fetal mice.
J. Exp. Med. 1974; 140: 1285-1302

Goidl, E.A., Klass, J., Siskind, G.W.
Ontogeny of B-lymphocyte function. II. Ability of endotoxin to increase the heterogeneity of affinity of the immune response of B-lymphocytes from fetal mice.
J. Exp. Med. 1976; 143: 1503-1520

Good, R.A., Varco, R.L.
A clinical and experimental study of agammaglobulinemia.
J. Lancet 1955; 75: 245-271

Gowans, J.L., Knight, E.J.,
The route of recirculation of lymphocytes in the rat.
Proc. Roy. Soc.(B) 1964; 159: 257-282

Green, D.R., Gold, J., St.Martin, S., Gershon, R., Gershon, R.K.
Microenvironmental immunoregulation: possible role of contrasuppressor cells in maintaining immune responses in gut associated lymphoid tissues.
Proc. Natl. Acad. Sci. USA 1982; 79: 889-892

Gruskay, F.L., Cooke, R.E.
The gastrointestinal absorption of unaltered protein in normal infants and in infants recovering from diarrhoea.
Pediatrics 1955; 16: 763-769

Guy-Grand, D., Griscelli, C., Vassalli, P.
The gut associated lymphoid system. Nature and properties of the large dividing cells.
Eur. J. Immunol. 1974; 4: 435-443

Guy-Grand, D., Griscelli, C., Vassalli, P.
The mouse gut T lymphocyte, a novel type of T-cell. Nature, origin and traffic in mice in normal and graft-versus-host conditions.
J.Exp. Med 1978; 148: 1661-1677

Haig, D.M., McKee, T.A., Jarrett, E.E.E., Woodbury, R., Miller, H.R.P.
Generation of mucosal mast cells is stimulated in vitro by factors derived from T-cells of helminth - infected rats.
Nature 1982; 300: 188-190

Hall, J.G., Smith, M.E.
Homing of lymph-borne immunoblasts to the gut.
Nature 1970; 226: 262-263

Hall, J.G., Parry, D.M., Smith, M.E.
The distribution and differentiation of lymph-borne immunoblasts after intravenous injection into syngeneic recipients.
Cell Tissue Kin. 1972; 5: 269-281

Halsey, J., Benjamin, D.
Induction of immunologic tolerance in nursing neonates by absorption of tolerogen from colostrum.
J. Immunol. 1976; 116: 1204-1207

Hamaoka, T., Katz, D.H., Benacerraf, B.
Radioresistance of carrier-specific helper thymus-derived lymphocytes in mice
Proc. Natl. Acad. Sci. USA 1972; 69: 3453-3458

Hanan, R., Oyama, J.
Inhibition of antibody formation in mature rabbits by contact with the antigen at an early age.
J. Immunol. 1954; 73: 49-53

Hanson, D.G., Vaz, N.M., Maia, L., Hornbrook, M., Lynch, J., Roy, C.
Inhibition of specific immune responses by feeding protein antigens.
Int. Arch. Allergy Appl. Immunol. 1977; 55: 526-532

Hanson, D.G., Vaz, N.M., Maia, L., Lynch, J.
Inhibition of specific immune responses by feeding protein antigens. III. Evidence against maintenance of tolerance to ovalbumin by orally induced antibodies.
J. Immunol. 1979; 123: 2337-2343

Hanson, D.G.
Ontogeny of orally induced tolerance to soluble proteins in mice. I. Priming and tolerance in newborns.
J. Immunol. 1981; 127: 1518-1524

Hanson, D.G., Miller, S.D.
Inhibition of specific immune responses by feeding protein antigens. V. Induction of the tolerant state in the absence of specific suppressor T-cells.
J. Immunol. 1982; 128: 2378-2381

Hanson, L.A., Carlsson, B., Cruz, J.R., Dahlgren, V., Garcia, B., Urrutia, J.J.
The enteromammaric axis of the secretory IgA system.
In: Ogra, P.L., Bienenstock, J., eds, The mucosal immune system in health and disease. Report of the 81st Conference on paediatric research. Columbus, Ohio, Ross Laboratories 1981: 30-33

Hardy, B., Mozes, E.
Expression of T-cell suppressor activity in the immune responses of newborn mice to a T-independent synthetic polypeptide.
Immunology 1978; 35: 757-762

Heremans, J.F.
Immunoglobulin A.
In: The antigens. Sela, M., ed., New York: Academic Press 1974: 365-522

Hjelm, H., Hjelm, K., Söquist, J.
Protein A from staphylococcus aureus. Its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins.
F.E.B.S. lett. 1972; 28: 73-76

Howard, J.G., Mitchison, N.A.
Immunological tolerance.
Progr. Allergy 1975; 18: 43-96

Hurme, M., Bang, B.E., Sihvola, M.
Genetic differences in the cyclophosphamide-induced immune suppression: weaker suppression of T-cell cytotoxicity by cyclophosphamide activated CBA mice.
Clin. Immunol. Immunopathol. 1980; 17: 38-42

Husband, A.J., Gowans, J.L.
The origin and antigen dependent distribution of IgA-containing cells in the intestine.
J. Exp. Med. 1978; 148: 1146-1160

Joel, D.D., Hess, M.W., Cottier, H.
Thymic origin of lymphoctyes in developing Peyer's patches of newborn mice.
Nature New Biol. 1971; 231: 24-25

Joel, D.D., Hess, M.W., Cottier, H.
Magnitude and pattern of thymic lymphocyte migration in neonatal mice.
J. Exp. Med. 1972; 135: 907-923

Kagnoff, M.F.

Effects of antigen feeding on intestinal and systemic immune responses. II. Suppression of delayed-type hypersensitivity reactions.

J.Immunol. 1978 a; 120: 1509-1513

Kagnoff, M.F.

Effects of antigen feeding on intestinal and systemic immune responses. III. Antigen specific serum mediated suppression of humoral antibody responses after antigen feeding.

Cell. Immunol. 1978 b; 40: 186-203

Kagnoff, M.F.

Effects of antigen feeding on intestinal and systemic immune responses. IV. Similarity between the suppressor factor in mice after erythrocyte lysate injection and erythrocyte feeding.

Gastroenterology 1980; 79: 54-61

Kamarck, M.E., Gottlieb, P.D.

Expression of thymocyte surface alloantigens in the fetal mouse thymus in vitro and in organ culture.

J. Immunol. 1977; 107: 407-415

Kaufmann, S.H.E., Hahn, H.

Regulation of delayed-type hypersensitivity to sheep red blood cells: demonstration of suppressor T-cells and a soluble suppressor factor.

Adv. Exp. Biol. Med. 1979; 114: 307-311

Kaufmann, S.H.E., Hahn, H., Diamantstein, T.

Relative susceptibilities of T-cell subsets involved in delayed-type hypersensitivity to sheep red blood cells to the in vitro action of 4-hydroxyperoxycyclophosphamide.

J. Immunol. 1980; 125: 1104-1108

Kealey, W.F.

Colonic lymphoid-glandular complex (microbursa).

Nature and morphology

J. Clin. Pathol. 1976; 29: 241-244

Kitamura, Y., Shimada, M., Hatanaka, K., Miyano, Y.
Development of mast cells from grafted bone marrow cells in irradiated mice.
Nature 1977; 268: 442-443

Kitamura, Y., Yokoyama, M., Matsuda, H., Ohno, T., Mori, K.J.
Spleen-colony-forming cell as a common precursor for tissue mast cells and granulocytes.
Nature 1981; 291: 159-160

Kojima, A., Tamura, S-I., Egashira, Y.
Regulatory role of suppressor T-cells in the expression of delayed-type hypersensitivity in mice. I. Properties of memory cells for delayed-type hypersensitivity against ovalbumin.
Cell. Immunol. 1979; 45: 61-73

Kontiainen, S., Howie, S., Maurer, P.H., Feldmann, M.
Suppressor cell induction in vitro. VI. Production of suppressor factors to synthetic polypeptides GAT and (T,G)-A-L from cells of responder and non-responder mice.
J. Immunol. 1979; 122: 253-259

Kuitunen, P., Visakorpi, J., Savilahti, E., Pelkonen, P.
Malabsorption syndrome with cows' milk intolerance. Clinical findings and course in 54 cases.
Arch. Dis. Child. 1975; 50: 351-356

L'age-Stehr, J., Diamantstein, T.
Studies on induction and control of cell mediated autoimmunity. II. Prevention of induction and activity of autoreactive T-cells by suppressor cells and by a suppressive serum factor.
Eur. J. Immunol 1978; 8: 624-628.

Lagrange, P.H., Mackaness, G.B., Miller, T.E.
Potentiation of T-cell mediated immunity by selective suppression of antibody formation with cyclophosphamide.
J. Exp. Med. 1974; 139: 1529-1539

Leblond, C.P.
The life history of cells in renewing systems.
Am. J. Anat. 1981; 160: 114-158

Leclerc, C., Juy, D., Bourgeois, E., Chedid, L.
In vivo regulation of humoral and cellular immune responses of mice by a synthetic adjuvant, N-acetyl-muramyl-L-alanyl-D-isoglutamine, muramyl dipeptide for MDP.
Cell. Immunol. 1979; 45: 199-206

Lederer, E.
Immunostimulation: recent progress in the study of natural and synthetic immunomodulators derived from the bacterial cell wall.
In: Fougereau, M., Dausset, J., eds. Progress in immunology IV, London New York: Academic Press 1980: 1194-1211

Levine, B., Vaz, N
The effect of combinations of inbred strain, antigen, and antigen dose on immune responsiveness and reagin production in the mouse.
Int. Arch. Allergy Appl. Immunol. 1970; 39: 156-171

Liew, F.Y., Sia, D.Y., Parish, C.R., McKenzie, I.F.C.
Major histocompatibility gene complex (MHC) - coded determinants on antigen-specific suppressor factor for delayed-type hypersensitivity and surface phenotypes of cells producing the factor.
Eur. J. Immunol. 1980; 10: 305-309

Lippard, V.W., Schloss, O.M., Johnson, P.A.
Immune reactions induced in infants by intestinal absorption of uncompletely digested cow's milk protein.
Am. J. Dis. Child. 1936; 51: 562-567

Löwy, I., Bona, C., Chedid, L.
Target cells for the activity of a synthetic adjuvant: muramyl dipeptide.
Cell. Immunol. 1977; 29: 195-199

Lyscom, N., Brueton, M.J.
Intraepithelial, lamina propria and Peyer's patch lymphocytes of the rat small intestine: isolation and characterisation in terms of immunoglobulin markers and receptors for monoclonal antibodies.
Immunology 1982; 45: 775-783

Lyscom, N., Brueton, M.J.
Study of the transfer of tolerance by mucosal intraepithelial and Peyer's patch lymphocytes.
Gut 1983; 24: A473

McWilliams, M., Phillips-Quagliata, J.M., Lamm, M.E.
Characteristics of mesenteric lymphnode cells homing to gut-associated lymphoid tissue in syngeneic mice.
J. Immunol. 1975; 115: 54-58

MacDonald, T.T., Ferguson, A.
Hypersensitivity reactions in the small intestine. II. Effects of allograft rejection and of graft-versus-host disease on mucosal architecture and lymphoid cell infiltrate.
Gut 1976; 17: 81-91

MacDonald, T.T., Ferguson, A.
Hypersensitivity reactions in the small intestine. III. Effects of allograft rejection and of graft-versus-host disease on epithelial cell kinetics.
Cell Tissue Kinet. 1977; 10: 301-312

MacDonald, T.T.
Immunosuppression caused by antigen feeding. I. Evidence for the activation of a feedback suppressor pathway in the spleens of antigen fed mice.
Eur. J. Immunol. 1982; 12: 767-773

Mattingly, J.A., Waksman, B.H.
Immunologic suppression after oral administration of antigen. I. Specific suppressor cells formed in rat Peyer's patches after oral administration of sheep erythrocytes and their systemic migration.
J. Immunol. 1978; 121: 1878-83

Mattingly, J.A., Kaplan, J.M., Janeway, L.A.,
Two distinct antigen specific suppressor factors induced by the oral administration of antigen.
J. Exp. Med. 1980; 152: 545-554

Mattingly, J.A., Waksman, B.H.
Immunologic suppression after oral administration of antigen. II. Antigen specific helper and suppressor factors produced by spleen cells of rats fed sheep erythrocytes.
J. Immunol. 1980; 125: 1044-1047

Mavromichalis, J., Brueton, M.J., McNeish, A.S., Anderson, C.M.
Evaluation of the intraepithelial lymphocyte count in the jejunum in childhood enteropathies.
Gut 1976; 17: 600-603

Mayrhofer, G.
Thymus dependent and thymus independent subpopulations of intestinal intraepithelial lymphocytes: a granular subpopulation of probable bone marrow origin and relationship to mucosal mast cells.
Blood 1980; 55: 532-535

Mayrhofer, G., Bazin, G.
Nature of the thymus dependency of mucosal mast cells. III. Mucosal mast cells in nude mice and nude rats and in a child with the Di George Syndrome.
Int. Arch. Allergy Appl. Immunol. 1981; 64: 320-331

Medawar, P.B.
Immunological tolerance.
Nature 1961; 189: 14-17

Melchers, F., von Boehmer, H., Phillips, R.A.
B-lymphocyte subpopulations in the mouse. Organ distribution and ontogeny of immunoglobulin-synthesizing and mitogen-sensitive cells.
Transplant. Rev. 1975; 25: 26-58

Melchers, F.
B-lymphocyte development in fetal liver. I. Development of reactivities to B-cell mitogens 'in vivo' and 'in vitro'.
Eur. J. Immunol. 1977; 7: 476-481

Michalek, S.M., Kiyono, H., Wannemuehler, M.J., Mosteller, L.M., McGhee, J.R.
Lipopolysaccharide (LPS) regulation of the immune response: LPS influence in oral tolerance induction.
J. Immunol. 1982; 128: 1992-1998

Miller, H.R.P., Jarrett, W.F.H.
Immune reactions in mucous membranes. I. Intestinal mast cell response during helminth expulsion in the rat.
Immunology 1971; 20: 277-288

Miller, J.F.A.P.
Immunologic function of the thymus
Lancet 1961; 2: 748-749

Miller, J.F.A.P., Vadas, M.A., Whitelaw, A., Gamble, J.
Role of major histocompatibility complex gene products in delayed-type hypersensitivity.
Proc. Natl. Acad. Sci. USA 1976; 73: 2486-2490

Miller, S.D., Hanson, D.G.
Inhibition of specific immune responses by feeding protein antigens. IV. Evidence for tolerance and specific active suppression of cell mediated immune responses to ovalbumin.
J. Immunol. 1979; 123: 2344-2350

Mitsuoka, A., Baba, M., Morikawa, S.
Enhancement of delayed hypersensitivity by depletion of suppressor T-cells with cyclophosphamide in mice.
Nature 1976; 262: 77-78

Mitsuoka, A., Morikawa, S., Baba, M., Harada, T.
Cyclophosphamide eliminates suppressor T-cells in age associated central regulation of delayed hypersensitivity in mice.
J. Exp. Med. 1979; 149: 1018-1028

Moore, M.A.S., Owen, J.J.T.
Experimental studies on the development of the thymus.
J. Exp. Med. 1967; 126: 715-726

Morris, C.K., Johnson, A.G.
Regulation of the immune system by synthetic polynucleotides. VII. Suppression induced by pretreatment with poly A:U.
Cell. Immunol. 1978; 39: 345-354

Morris, I.G.
Gamma globulin absorption in the newborn.
In: Code, C.F., Heidel, W., eds, Handbook of Physiology, Sect. 6 Alimentary Canal, Vol. 2
Williams and Wilkins, Baltimore 1968: 1491-1512

Mosier, D.E., Johnson, B.M.
Ontogeny of mouse lymphocyte function. II. Development of the ability to produce antibody is modulated by T-lymphocytes.
J. Exp. Med. 1975; 141: 216-226

Mosier, D.E., Mathieson, B.J., Campbell, P.S.
Ly-phenotype and mechanism of action of mouse neonatal suppressor T-cells.
J. Exp. Med. 1977; 146: 59-73

Mowat, A.McI.
Induction and expression of delayed hypersensitivity in the small intestine.
Ph.D. Thesis, University of Edinburgh 1981

Mowat, A.McI., Ferguson, A.
Hypersensitivity in the small intestinal mucosa. V. Induction of cell mediated immunity to a dietary antigen.
Clin. Exp. Immunol. 1981; 43: 574-582

Mowat, A.McI., Ferguson, A.
Intraepithelial lymphocyte count and crypt hyperplasia measure the mucosal component of the graft-versus-host reaction in mouse small intestine.
Gastroenterology 1982; 83: 417-423

Muckerheide, A., Pesce, A.J., Michael, J.G.
Modulation of the IgE immune response to BSA by fragments of the antigen. I. Suppression by free fragments and by fragments conjugated to homologous gamma-globulin
Cell. Immunol. 1981; 59: 392-398

Murgita, A., Wigzell H.
Regulation of immune functions in the fetus and newburn.
Prog. Allergy 1981; 29: 54-133

Murray, M., Miller, H.R.P., Jarrett, W.F.H.
The globule leucocyte and its derivation from the subepithelial mast cell.
Lab. Invest. 1968; 19: 222-234

Nagatomi, H., Ogita, T., Okudaira, H., Mitzushima, Y.
Antigen-binding activity and allergenicity of heterologous gamma globulin absorbed from the rectum.
Int. Arch. Allergy Appl. Immunol. 1980; 63: 340-343

Neveu, P.G., Borduas, A.G.
Carrier function in immune deviation.
J. Immunol. 1974; 112: 1264-1266

Ngan, J., Kind, L.
Suppressor T-cells for IgE and IgG in Peyer's patches of mice made tolerant by the oral administration of ovalbumin.
J. Immunol. 1978; 120: 861-865

Nossal, G.J.V., Pike, B.L.
Studies on the differentiation of B-lymphocytes in the mouse.
Immunology 1973; 25: 33-45

Nossal, G.J.V., Pike, B.L.
Evidence for the clonal abortion theory of B-lymphocyte tolerance.
J. Exp. Med. 1975; 141: 904-917

Nossal, G.J.V., Pike, B.L.
Antibody receptor diversity and diversity of signals.
In: Fougereau, M., Dausset, J., eds., Progress in immunology IV, London: Academic Press 1980; 136-152

Olson, C.E., Levy, D.A.
Thymus dependency of the mast cell response to nippostrongylus brasiliensis in mice.
Fed. Proc. 1976; 35: 491

Ottaway, C.A., Parrott, D.M.V.
Regional blood flow and the localisation of lymphoblasts in the small intestine of the mouse. I. Examination of normal small intestine.
Immunology 1980; 41: 955-961

Otto, H.F.
The interepithelial lymphocytes of the intestinum: morphological observations and immunological aspects of intestinal enteropathy.
Curr. Topics Pathol. 1973; 57: 81-121

Owen, J.J.T., Raff, M.C.
Studies on the differentiations of thymus-derived lymphocytes.
J. Exp. Med. 1970; 132: 1216-1232

Owen, J.J.T., Raff, M.C., Cooper, M.D.
Studies on the generation of B-lymphocytes in the mouse embryo.
Eur. J. Immunol. 1975; 5: 468-473

Owen, R.D.
Immunogenetic consequences of vascular anastomoses between bovine twins.
Science 1945; 102: 400-401

Owen, R.L., Jones, A.L.
Epithelial cell specialisation within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles.
Gastroenterology 1974; 66: 189-203

Owen, R.L.
Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study.
Gastroenterology 1977; 72: 440-451

Pabst, M.J., Johnston, R.B.
Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide or lipopolysaccharide.
J. Exp. Med. 1980; 151: 101-114

Parks, D.E., Doyle, M.V., Weigle, W.O.
Induction and mode of action of suppressor cells generated against human gamma globulin. I. An immunologically unresponsive state devoid of demonstrable suppressor cells.
J. Exp. Med. 1978; 148: 625-638

Parks, D.E., Weigle, W.O.
Current perspectives on the cellular mechanisms of immunologic tolerance.
Clin. Exp. Immunol. 1980 a; 39: 257-262

Parks, D.E., Weigle, W.O.
Maintenance of immunologic unresponsiveness to human gamma globulin: evidence for irreversible inactivation in B-lymphocytes.
J. Immunol. 1980 b; 124: 1230-1236

Parrott, D.M.V., De Sousa, M.A.B.
Thymus dependent and thymus independent populations: origin, migratory patterns and lifespan.
Clin. Exp. Immunol. 1971; 8: 663-684

Parrott, D.M.V.
The gut associated lymphoid tissues and gastrointestinal immunity.
In: Ferguson, A., MacSween, R.N.M., eds, Immunological aspects of the liver and gastrointestinal tract.
Lancaster: M.T.P. Press Ltd 1976: 1-32

Parrott, D.M.V., Tait, C., MacKenzie, S., Mowat, A.McI., Davies, M.D.J., Micklem, H.S.
Analysis of the effector functions of different populations of mucosal lymphocytes.
Ann. N.Y. Acad. Sci. 1983; 409: 307-320

Perrotto, J.L., Hang, L.M., Isselbacher, K.J., Warren, K.S.
Systemic cellular hypersensitivity induced by an intestinally absorbed antigen.
J. Exp. Med. 1974; 140: 296-299.

Phillips, A.D., Rice, S.J., France, N.E., Walker-Smith, J.A.
Small intestinal intraepithelial lymphocyte levels in cows milk protein intolerance.
Gut 1979; 20: 509-512

Phillips, R.A., Melchers, F.
Appearance of functional lymphocytes in fetal liver.
J. Immunol. 1976; 117: 1099-1103

Pierce, N.F., Gowans, J.L.
Cellular kinetics of the intestinal immune response to cholera toxoid in rats.
J. Exp. Med. 1975; 142: 1550-1563

Pierce, N.F., Sack, R.B., Sircar, B.K.
Immunity to experimental cholera. III. Enhanced duration of protection after sequential parenteral-oral administration of toxoid to dogs.
J. Infect. Dis. 1977; 135: 888-896

Polak, L., Geleick, H., Turk, J.L.
Reversal by cyclophosphamide of tolerance in contact sensitisation: tolerance induced by prior feeding with DNCB.
Immunology 1975; 28: 939-942

Ptak, W., Skowron-Cendrzak, A.
Fetal suppressor cells. Their influence on the cell mediated immune responses.
Transplantation 1977; 24: 45-51

Rabinowitz, S.G.
Measurement and comparison of the proliferative and antibody responses of neonatal, immature and adult murine spleen cells to T-dependent and T-independent antigens.
Cell. Immunol. 1976; 21: 201-216

Raff, M.C.
Evidence for a subpopulation of mature lymphocytes within mouse thymus.
Nature New Biol. 1971; 229: 182-184

Raff, M.C.
T and B-lymphocytes and immune responses.
Nature 1973; 242: 19-23

Raff, M.C., Owen, J.J.T., Cooper, M.D., Lawton, A.R., Megson, M., Gathings, W.E.
Differences in susceptibility of mature and immature mouse B-lymphoctyes to anti-immunoglobulin induced immunoglobulin suppression in vitro.
Possible implications for B-cell tolerance to self.
J. Exp. Med. 1975; 142: 1052-1064

Raff, M.C., Megson, M., Owen, J.J.T., Cooper, M.D.
Early production of intracellular IgM by B-lymphocyte precursors in mouse.
Nature 1976; 259: 244-246

Ramshaw, I.A., Bretscher, P.A., Parish, C.R.
Regulation of the immune response. I. Suppression of delayed-type hypersensitivity by T-cells from mice expressing humoral immunity.
Eur. J. Immunol. 1976; 6: 674-679

Reilly, R.W., Kirsner, J.B.
Runt intestinal disease.
Lab. Invest. 1965; 14: 102-107

Richman, L.K., Chiller, J.M., Brown, W.R., Hanson, D.G., Vaz, N.
Enterically induced immunologic tolerance. I. Induction of suppressor T-lymphocytes by intragastric administration of soluble proteins.
J. Immunol. 1978; 121: 2429-2934

Richman, L.K., Klingenstein, R.J., Richman, J.A., Strober, W., Berzofsky, J.A.
The murine Kupffer cell. I. Characterisation of the cell serving accessory function in antigen-specific T-cell proliferation.
J. Immunol. 1979; 123: 2602-2609

Richman, L.K., Graeff, A.S., Yarchoan, R., Strober, W.
Simultaneous induction of antigen specific IgA helper T-cells and IgG suppressor T-cells in the murine Peyer's patch after protein feeding.
J. Immunol. 1981; 126: 2079-2083

Roberts-Thomson, I.C., Mitchell, G.F.
Giardiasis in mice. I. Prolonged infections in certain mouse strains and hypothymic (nude) mice.
Gastroenterology 1978; 75: 42-46

Röllinghoff, M., Starzinski-Powitz, A., Pfizenmaier, K., Wagner, H.
Cyclophosphamide-sensitive T-lymphocytes suppress the in vivo generation of antigen-specific cytotoxic T-lymphocytes.
J. Exp. Med. 1977; 145: 455-459

Rose, M.L., Parrott, D.M.V., Bruce, R.G.
Migration of lymphoblasts to the small intestine. II. Divergent migration of mesenteric and peripheral immunoblasts to sites of inflammation in the mouse.
Cell. Immunol. 1976; 27: 36-46

Rothberg, R.M., Farr, R.S.
Anti-bovine serum albumin and anti-alpha lactalbumin in the sera of children and adults.
Pediatrics 1965; 35: 571-575

Rothberg, R.M., Rieger, C.H.L., Silverman, G.A., Peri, B.A.
Antigen uptake and antibody production in the human newborn.
In: Ogra, P.L., Bienenstock, J., eds, The mucosal immune system in health and disease. Report of the 81st Ross Conference on Paediatric Research, Columbus, Ohio, Ross Laboratories 1981: 57-62

Rudzik, O., Bienenstock, J.
Isolation and characteristics of gut mucosal lymphoctyes.
Lab. Invest. 1974; 30: 260-266

Rudzik, O., Clancy, R.L., Perey, D.Y.E., Bienenstock, J.
Repopulation with IgA-containing cells of bronchial and intestinal lamina propria after transfer of homologous Peyer's patches and bronchial lymphocytes.
J. Immunol. 1975; 114: 1599-1604

Ruitenberg, E.J., Elgersma, A.
Absence of intestinal mast cell response in congenitally athymic mice during Trichinella spiralis infection.
Nature 1976; 264: 258-260

Scott, D.W., Layton, J.E., Nossal, G.J.V.
Role of IgD in the immune response and tolerance. I Anti-delta-pretreatment facilitates tolerance induction in adult B-cells in vitro.
J. Exp. Med. 1977; 146: 1473-1483

Schrader, J.W., Scollay, R., Battye, F.
Intramucosal lymphocytes of the gut. Lyt-2 and Thy-1 phenotype of the granulated cells and evidence for the presence of both T-cells and mast cell precursors.
J. Immunol. 1983; 130: 558-564

Selby, W.S., Janossy, G., Goldstein, G., Jewell, D.P.
T-lymphocyte subsets in human intestinal mucosa: the distribution and relationship to MHC derived antigens.
Clin. Exp. Immunol. 1981; 44: 453-458

Sherr, D.H., Szewczuk, M.R., Cusano, A., Rapaport, W., Siskind, G.W.
Ontogeny of B-lymphocyte function. IX. Difference in the time of maturation of the capacity of B-lymphocytes from fetal and neonatal mice to produce a heterogenous antibody response to thymic-dependent and thymic-independent antigens.
Immunology 1979; 36: 891-907

Shiho, O., Takaoki, M., Nakagawa, Y., Arakawa, M., Takeuchi, M.
Carrier-induced T-helper and T-suppressor cells involved in the regulation of hapten-specific DTH responses.
J. Immunol. 1982; 129: 966-970

Shimamura, T., Hashimoto, K., Sasaki, S.
Feedback suppression of the immune response in vivo. I. Immune B-cells induce antigen-specific suppressor T-cells.
Cell. Immunol. 1982; 68: 104-113

Silver, J., Benacerraf, B.
Dissociation of T-cell helper function and delayed hypersensitivity.
J. Immunol. 1974; 113: 1872-1875

Simonsen, M.
Graft-versus-host reactions. Their natural history and applicability as tools of research.
Progr. Allergy 1962; 6: 349-476

Singal, D.P., O'Neill, M., Clancy, R., Bienenstock, J.
Functional T-cells in rabbit gut mucosal lymphocytes.
Gut 1976; 17: 325-327

Singh, J.N., Sabbadini, E., Sehon, A.H.
Cytotoxicity in graft-versus-host reaction. I. Role of donor and host spleen cells.
J. Exp. Med. 1972; 136: 39-48

Siskind, G.W.

Ontogeny of the immune system.
In: Segre, D., Smith, L., eds., Immunological aspects of ageing.
New York: Marcel Dekker Inc., 1981: 21-39

Slavin, R.E., Santos, G.W.

The graft-versus-host reaction in man after bone marrow transplantation: pathology, pathogenesis, clinical features and implications.
Clin. Immunol. Immunopathol. 1973; 1: 472-498

Spear, P.G., Wang, A-L., Rutishauser, U., Edelman, G.M.

Characterisation of splenic lymphoid cells in fetal and newborn mice.
J. Exp. Med. 1973; 138: 557-573

Spear, P.G., Edelman, G.M.

Maturation of the humoral immune response in mice.
J. Exp. Med. 1974; 139: 249-263

Sprent, J., Anderson, R.E., Miller, J.F.A.P.

Radiosensitivity of T and B-lymphocytes. II. Effects of irradiation on response of T-cells to alloantigens.
Eur. J. Immunol. 1974; 4: 204-210

Sprent, J.

Fate of H2-activated T-lymphocytes in syngeneic hosts. I. Fate in lymphoid tissues and intestines traced with ^{3}H-thymidine, ^{125}I-deoxyuridine and ^{51}chromium.
Cell. Immunol. 1976; 21: 278-302

Stenqvist, H.

Die 'Zellenwanderung' durch das Darmepithel.
Anat. Anz. 1934; 78: 68-79

Stephenson, P., Dorsch, S., Roser, B., Godden, U., Herbert, J.
The identity of suppressor cells in neonatal tolerance.
Transplant. Proc. 1983; 15: 850-852

Stevens, F.A.
Status of poison ivy extracts.
J.A.M.A. 1945; 127: 912-921

Stokes, C.R., Swarbrick, E.T., Soothill, J.F.
Genetic differences in immune exclusion and partial tolerance to ingested antigens.
Clin. Exp. Immunol. 1983; 52: 678-684

Strober, W., Richman, L.K., Elson, C.O.
The regulation of gastrointestinal immune responses.
Immunology today 1981; 2: 156-162

Stutman, O., Good, R.A.
Immunocompetence of embryonic hematopoietic cells after traffic to thymus.
Transplant. Proc. 1971; 3: 923-925

Stutman, O.
Intrathymic and extrathymic T-cell maturation.
Immunol. Rev. 1978; 42: 138-184

Stutman, O.
Thymic hormones, T-cell development and ageing.
In: Segre, D., Smith, L., eds., Immunological aspects of ageing.
New York: Marcel Dekker Inc., 1981: 333-370

Swarbrick, E.T.
Antigen handling by the gut.
M.D. Thesis, University of London 1979

Swarbrick, E.T., Stokes, C.R., Soothill, J.F.
Absorption of antigens after oral immunisation and the simultaneous induction of specific systemic tolerance.
Gut 1979; 20: 121-125

Tada, T., Okumura, K.
The role of antigen-specific T-cell factors in the immune response.
Adv. Immunol. 1979; 28: 1-87

Tada, T., Taniguchi, M., Takemori, T.
Properties of primed suppressor T-cells and their products.
Transplant. Rev. 1975; 26: 106-129

Taliaferro, W.H., Taliaferro, L.G., Jaroslow, B.N, eds.
Radiation and immune mechanisms
New York: Academic Press 1964

Taussig, M.J.
Antigen-specific T-cell factors.
Immunology 1980; 41: 759-787

Tew, K.D., Taylor, D.M.
Studies with cyclophosphamide labelled with phosphorus 32: nucleic acid alkylation and its effects on DNA synthesis in rat tumour and normal tissue.
J. Natl. Cancer Inst. 1977; 58: 1413-1419

Thomas, H.C., Parrott, D.M.V.
The induction of tolerance to a soluble protein antigen by oral administration.
Immunology 1974; 27: 631-639

Thomas, H.C., Ryan, C.J., Benjamin, I.S., Blumgart, L.H., MacSween, R.N.M.
The immune response in cirrhotic rats. The induction of tolerance to orally administered protein antigens.
Gastroenterology 1976; 71: 114-117

Timonen, T., Ortaldo, J.R., Herberman, R.B.
Characteristics of human large granular lymphocytes and relationship to natural killer and K-cells.
J. Exp. Med. 1981; 153: 569-582

Titus, R.G., Chiller, J.M.
Orally induced tolerance: definition at the cellular level.
Int. Arch. Allergy Appl. Immunol. 1981; 65: 323-328

Tomasi, T.B., Tan, E.M., Solomon, A., Prendergast, R.A.
Characteristics of an immune system common to certain external secretions.
J. Exp. Med. 1965; 121: 101-124

Tomasi, T.B.
Oral tolerance.
Transplantation 1980; 29: 353-356

Treiber, W., Lapp, W.S.
Graft-versus-host induced immunosuppression. The effect of antigenic stimulation on cell mediated and humoral immune responses.
Transplantation 1973; 16: 211-216

Treiber, W., Lapp, W.S.
Experimental stimulation of cell mediated immunity without concomitant stimulation of humoral immunity in graft versus host immunosuppresed mice.
Transplantation 1976; 21: 391-398

Triger, D.R., Cynamon, M.H., Wright, R.
Studies on hepatic uptake of antigen. Comparison of inferior vena cava and portal vein routes of immunisation.
Immunology 1973; 25: 941-950

Turk, J.L., Parker, D., Poulter, L.W.
Functional aspects of the selective depletion of lymphoid tissue by cyclophosphamide.
Immunology 1972; 23: 493-501

Uhlenhuth
Neuer Beitrag zum spezifischen Nachweis von Eiereiweiss auf biologischem Wege.
Dtsch. Med. Wschr. 1900; 26: 734-735

Van Asperen, P.P., Kemp, A.S., Mellis, C.M.
Immediate food hypersensitivity reactions on the first known exposure to the food.
Arch. Dis. Child. 1983; 58: 253-256

Vaz, N., Maia, L., Hanson, D. G., Lynch, J.
Inhibition of homocytotropic antibody responses in adult inbred mice by previous feeding of the specific antigen.
J. Allergy Clin. Immunol. 1977; 60: 110-115

Vitetta, E.S., Uhr, J.
Immunoglobulin receptors revisited.
Science 1975; 189: 964-969

Vitoria, J.C., Camarero, C., Sojo, A., Ruiz, A., Rodriguez-Soriano, J.
Enteropathy related to fish, rice and chicken.
Arch. Dis. Child. 1982; 57: 44-48

Vives, J., Parks, D.E., Weigle, W.O.
Immunologic unresponsiveness after gastric administration of human gamma globulin: antigen requirements and cellular parameters.
J. Immunol. 1980; 125: 1811-1816

Voller, A., Bidwell, D.E., Bartlett, A., eds.
The enzyme linked immunosorbent assay (ELISA). A guide with abstracts of microplate applications (1979). Nuffield Laboratories of Comparative Medicine, The Zoological Society of London, Regent's Park, London,
(available from Dynatech, Europe, Borough House, Rue de Pre, Guernsey, United Kingdom)

Waksman, B.H.
The homing pattern of thymus derived lymphocytes in calf and neonatal mouse Peyer's patches.
J. Immunol. 1973; 111: 878-884

Waksman, B.H., Ozer, H.
Specialised amplification elements in the immune system.
The role of nodular lymphoid organs in the mucous membranes.
Progr. Allergy 1976; 21: 1-113

Waksman, B.H.
Tolerance, the thymus and suppressor T-cells.
Clin. Exp. Immunol. 1977; 28: 363-374

Waksman, B.H.
Cellular hypersensitivity and immunity: conceptual changes in last decade.
Cell. Immunol. 1979; 42: 155-169

Walker, W.A., Isselbacher, K.J.
Uptake and transport of macromolecules by the intestine: possible role in clinical disorders.
Gastroenterology 1974; 67: 531-550

Walker, W.A.
Intestinal transport of macromolecules.
In: Johnson, L.R., ed., Physiology of the gastrointestinal tract, New York: Raven Press 1981: 1271-1289

Wannemuehler, M.J., Kiyono, H., Babb, J.L., Michalek, S.M., McGhee, J.R.
Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germ free mice to sensitivity to oral tolerance induction.
J. Immunol. 1982; 129: 959-965

Warner, N.L., Szenberg, A., Burnet, F.M.
The immunologic role of different lymphoid organs in the chicken. I. Dissociation of immunologic responsiveness.
Aust. J. Exp. Biol. 1962; 40: 373-387

Warshaw, A.L., Walker, W.A.
Protein uptake by the intestine: evidence for absorption of intact macromolecules.
Gastroenterology 1974; 66: 987-992

Waters, C., Pilarski, L., Wegmann, T., Diener, E.
Tolerance induction during ontogeny. I. Presence of active suppression in mice rendered tolerant to human gamma-globulin in utero correlates with the breakdown of the tolerant state.
J. Exp. Med. 1979; 149: 1134-1151

Weigle, W.O.
Immunologic tolerance and immunopathology.
Hosp. Pract. 1974; 12: 71-80

Weisz-Carrington, P., Roux, M.E., McWilliams, M.
Phillips-Quagliata, J.M., Lamm, M.E.
Hormonal induction of the secretory immune system in the mammary gland.
Proc. Natl. Acad. Sci. USA 1978; 75: 2928-2932

Wells, H.G., Osborne, T.B.
The biological reactions to vegetable proteins. I. Anaphylaxis.
J. Infect. Dis. 1911; 8: 66-124

Wu, A.M., Till, J.E., Siminovitch, L., McCullogh, E.A.
A cytological study of the capacity for differentiation of normal hematopoietic colony-forming cells.
J. Cell Physiol. 1967; 69:177-184

Zinkernagel, R.M. Doherty, P.C.
Major transplantation antigens, viruses and specificity of surveillance T-cells.
Contemp. Top. Immunol. 1977; 7: 179-220

I herewith declare that the thesis has been composed by myself and that the presented work is my own.
Members of the research group who contributed to this work have been acknowledged.

Stephan Strahl

21/12/1983

Immunology 1983 **49** 451

Immunological responses to fed protein antigens in mice

II. ORAL TOLERANCE FOR CMI IS DUE TO ACTIVATION OF CYCLOPHOSPHAMIDE-SENSITIVE CELLS BY GUT-PROCESSED ANTIGEN

S. STROBEL, A. McI. MOWAT,* HAZEL E. DRUMMOND, MAUREEN G. PICKERING & ANNE FERGUSON *Gastro-Intestinal Unit, University of Edinburgh and Western General Hospital, Edinburgh and* **Department of Bacteriology and Immunology, Western Infirmary, Glasgow*

Accepted for publication 2 February 1983

Summary. Mice fed ovalbumin develop specific systemic hyporesponsiveness. This oral tolerance is abrogated by cyclophosphamide pretreatment, and the mechanism of abrogation could be either via T suppressor cells or via damage to the gut epithelium. A serum transfer protocol was used to examine the site of action of cyclophosphamide in this system. Serum was collected from ovalbumin-fed mice and transferred into recipients which were then parenterally immunized with ovalbumin in Freund's complete adjuvant. Serum transfer suppressed the delayed-type hypersensitivity (DTH) responses but not the antibody responses of the recipients. Cyclophosphamide pretreatment (100 mg/kg) of recipients (but not of donors) abrogated this suppressor effect. Parenteral administration of ovalbumin in a range of doses did not induce immunological hyporesponsiveness. It is suggested that absorption across the gut mucosa leads to generation of fragments of ovalbumin that induce suppressor cells selective for DTH.

Correspondence: Dr Stephan Strobel, Gastro-Intestinal Unit, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh.

0019-2805/83/0700-0451$02.00
© 1983 Blackwell Scientific Publications

INTRODUCTION

Induction of systemic tolerance by feeding protein antigens was first described 70 years ago (Wells & Osborne, 1911) and has been confirmed in many recent experiments. The mechanisms responsible for this oral tolerance involving both humoral and cell-mediated immunity, remain controversial. Thus, in different systems, there is evidence implicating immune complexes (André *et al.*, 1975) anti-idiotypic antibody (Kagnoff, 1980) and specific suppressor T cells (Ngan & Kind, 1978; Miller & Hanson, 1979). We have reported abrogation of oral tolerance in mice pretreated with cyclophosphamide before being fed the protein antigen ovalbumin (Mowat *et al.*, 1982), and we postulated that this modulation of tolerance was related to the effect of cyclophosphamide on suppressor T cells (Röllinghoff *et al.*, 1977; Schwartz, Askenase & Gershon, 1978). However since cyclophosphamide also acts on rapidly dividing cells such as intestinal enterocytes, it is equally possible that the modulation of oral tolerance by cyclophosphamide was due to alterations in the digestion or processing of protein antigen by the gut.

We reasoned that it should be feasible to examine the effects of 'gut processing' on the immunogenicity or tolerogenicity of fed antigen by comparing the

in-vivo effects of antigen which had been absorbed across the gut into the serum of mice, with the effects of administration of similar amounts of native antigen. A serum transfer protocol was used, in which serum was collected 1 h after mice had been fed the protein antigen ovalbumin, and then transferred intraperitoneally into syngeneic recipients. The immunological properties (tolerogenic or immunogenic) of the antigen in the transferred serum were then assessed by measurement of humoral and cell-mediated immunity in the recipients after a subsequent parenteral injection of ovalbumin. We also investigated the site of action of cyclophosphamide in modulating oral tolerance by separately treating donors and recipients of serum with cyclophosphamide. If cyclophosphamide acts at the level of the intestine, pretreatment of ovalbumin fed donors should alter any immunological effects which the transferred serum has, whereas if cyclophosphamide exerts its effect by inhibiting suppressor cells, then cyclophosphamide treatment of serum recipients should alter their immune responses.

MATERIALS AND METHODS

Animals

Male and female BALB/c mice, aged 6–8 weeks were used as donors of serum. Female BALB/c mice of the same age were used as recipients. Details of the various experimental groups are given in Table 1.

Antigens

Ovalbumin (Sigma Fraction V) was dissolved in distilled water for use.

Cyclophosphamide

Cyclophosphamide (Endoxana WB Pharmaceuticals) was dissolved in distilled water for use and mice were given 100 mg/kg by intraperitoneal injection, 2 days before ovalbumin feeding or serum transfer.

Collection and transfer of serum

20 donor mice per group were fed either 25 mg ovalbumin in 0·2 ml of water or 0·2 ml water, by intragastric tube and were bled out from the axillary vein 1 hr later. Sera from each group were pooled and 0·8 ml transferred intraperitoneally into each of six recipient mice. All recipients were immunized 1 week later with 100 μg ovalbumin in Freund's complete adjuvant (FCA; H37Ra, Difco Ltd) injected into one footpad.

Serum antibody levels

Mice were bled from the retro-orbital plexus, 14 and 21 days after immunization, and the sera tested for haemagglutinating antibodies to ovalbumin as de-

Table 1. Details of experimental protocol and treatment groups

Recipient mice groups	Treatment of serum donors		Treatment of recipients		
	Day −2	Day 0 (feed)	Day −2	Day 0	Days 7–28 (All groups)
A	H_2O	H_2O	H_2O	serum i.p.	
B	H_2O	OVA	H_2O	serum i.p.	
C	CY	H_2O	H_2O	serum i.p.	Day 7—100 μg OVA i.d. via footpad.
D	CY	OVA	H_2O	serum i.p.	
E	H_2O	H_2O	CY	serum i.p.	
F	H_2O	OVA	CY	serum i.p.	Day 21 and Day 27—bleed
G	—	—	—	saline i.v.	for antibodies.
H	—	—	—	1 μg OVA i.v.	
I	—	—	—	10 μg OVA i.v.	Day 28—skin test for DTH.
J	—	—	—	100 μg OVA i.v.	
K	—	—	—	10 μg OVA i.p.	
L	—	—	—	100 μg OVA i.p.	

Abbreviations: OVA, ovalbumin; CY, cyclophosphamide; i.p., intraperitoneal; i.v., intravenous; i.d., intradermal; DTH, delayed-type hypersensitivity.

scribed previously (Mowat & Ferguson, 1981). Results were expressed as the mean $\log_{10}$ antibody titre.

Delayed-type hypersensitivity responses

Three weeks after immunization, recipients were tested for systemic delayed-type hypersensitivity (DTH) by an intradermal footpad test. Footpad thicknesses were measured by skin calipers immediately before, and 24 hr after, an intradermal injection of 100 μg ovalbumin in saline. Ovalbumin-specific increments in footpad thickness were obtained by subtracting the response of immunized animals to saline alone.

Pretreatment of mice with parenteral injections of ovalbumin

For these experiments eight mice/group were given 1, 10 or 100 μg ovalbumin in 0·2 ml saline either intravenously or intraperitoneally, 1 week before immunization with ovalbumin in FCA as above. Control mice received 0·2 ml saline alone.

Statistics

Results are expressed as means $\pm$ one standard deviation of the mean, and groups were compared by Student's *t* test.

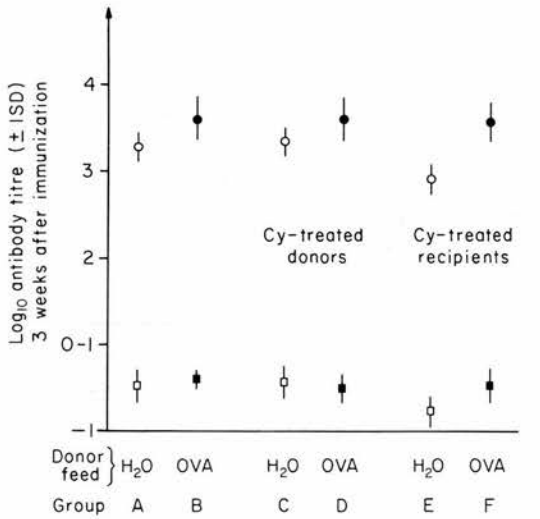


Figure 1. Effects of cyclophosphamide pretreatment of donors and recipients on serum antibody responses in recipients (Groups A to F), 3 weeks after parenteral immunization with ovalbumin in Freund's complete adjuvant; mean $\pm$ 1 standard deviation. *Circles* represent total Ig-titres, *squares* represent IgM (= 2-mercaptoethanol-sensitive) antibody titres.

RESULTS

Serum antibodies in recipients before parenteral immunization

All recipient mice were bled immediately prior to parenteral immunization with ovalbumin in FCA. None of the sera contained detectable ovalbumin-specific antibodies.

Effects of serum transfer on systemic immune responses of recipients

Analysis of the results in recipients of serum from water-fed donors (Group A) and ovalbumin-fed donors (Group B) showed that serum transfer had no effect on systemic humoral immune responses, the two groups having identical antibody levels, both total and IgM, at 2 and 3 weeks after immunization. Results for a typical experiment are illustrated in Fig. 1, Groups A and B. In contrast, the mice receiving serum from ovalbumin-fed donors had significant suppression of cell-mediated immunity (CMI) responses (Fig. 2, Group B) with 67% suppression ($P < 0{\cdot}02$) when compared with mice receiving serum from water-fed donors. This experiment was performed three times with identical results.

Effects of cyclophosphamide pretreatment of donors on immune responses of recipients

The serum from cyclosphosphamide-pretreated, ovalbumin-fed donors induced a similar degree of tolerance for CMI in the recipients (Group D, Fig. 2, 87% suppression when compared with Group C, $P < 0{\cdot}01$). Serum antibody levels were identical to Groups A and B (Fig. 1).

Effects of cyclophosphamide pretreatment of recipients on their immune responses

Recipient groups E and F received cyclophosphamide 2 days before serum transfer. As shown in Fig. 2, cyclophosphamide pretreatment abrogated the immunological tolerance for CMI which occurred when similar serum was transferred to saline-pretreated recipients (Group F compared with Group B). No significant effects on serum antibody responses were found. Although the titres of antibody in Group F were consistently higher than in Group E in three

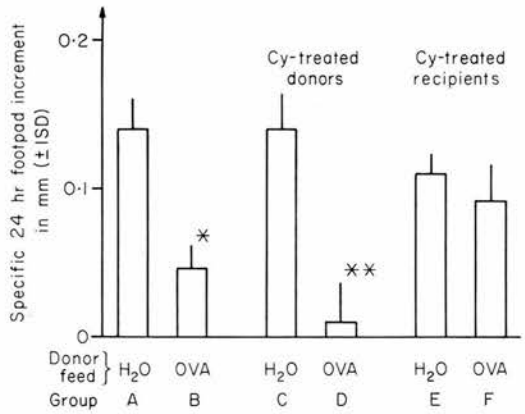


Figure 2. Effects of cyclophosphamide pretreatment of donors and recipients on DTH responses in recipients (Groups A to F), 3 weeks after parenteral immunization with ovalbumin in Freund's complete adjuvant; mean ± 1 standard deviation. (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$).

separate experiments, this difference never reached statistical significance.

Effects of parenteral ovalbumin on subsequent immune responses of recipients

Unpublished experiments with radioimmunoassay (Hanson, personal communication) and enzyme-linked immunoassay techniques (Strobel, unpublished) indicated that the amount of ovalbumin present in serum after feeding was likely to be in the range 0·01–1% of the ingested dose/ml mouse serum. Therefore, doses of 1, 10 and 100 μg native ovalbumin were administered intravenously or intraperitoneally into recipient mice which were then immunized parenterally with ovalbumin in Freund's complete adjuvant, and tested for systemic immunity in the same way as serum recipients (Groups H to L). As illustrated in Table 2, native ovalbumin in the doses used had no significant effects on the humoral or CMI responses to a subsequent parenteral immunization with ovalbumin.

DISCUSSION

The transfer of tolerance by serum from protein-fed mice has not previously been reported. In mice fed repeated doses of sheep red blood cells, the tolerogenic factors described in serum appear to reflect immune responses by the animal to the fed antigen, for transferred sera used in experiments with sheep red blood cell contain antibodies (Kagnoff, 1978), immune complexes (André *et al.*, 1975) and a serum factor which may be anti-idiotypic antibody (Kagnoff, 1980).

In the interpretation of their experiments on the induction of tolerance to oxazolone, Asherson, Perera & Thomas (1979) suggested that differences in the quantity of systemically available antigen could account for the contrasting effects of oral and parenteral administration of antigen. However it is unlikely that results in our experiments are due to the amount of ovalbumin absorbed, since parenteral administration of ovalbumin over a hundred-fold range of doses had no tolerizing effect on the immune responses of recipients. It is more likely that the tolerogenic properties of the serum from protein-fed mice are related to physico-chemical alterations in the protein

Table 2. Effects of parenteral ovalbumin injections on subsequent systemic immune responses, 3 weeks after parenteral immunization with ovalbumin in Freund's complete adjuvant

Group	Ovalbumin dose	Route	n*	Log_{10} antibody titres (mean ± 1 SD)	n*	Specific 24 hr footpad increment in mm (mean ± 1 SD)
G	Saline	I.V.	6	2·84 ± 0·22	5	0·17 ± 0·02
H	1 μg	I.V.	6	3·14 ± 0·20	5	0·19 ± 0·02
I	10 μg	I.V.	6	2·96 ± 0·18	5	0·20 ± 0·04
J	100 μg	I.V.	7	3·11 ± 0·25	6	0·17 ± 0·02
K	10 μg	I.P.	6	2·96 ± 0·15	5	0·15 ± 0·03
L	100 μg	I.P.	6	3·01 ± 0·23	5	0·16 ± 0·02

* Experimental animals/group.

occurring in the intestine. After the feeding of ovalbumin, large amounts of antigen free of aggregates are found in the serum (Swarbrick, 1979) and such deaggregated proteins are known to be extremely effective in inducing both oral and parenteral tolerance (Vives, Parks & Weigle, 1980; Parks & Weigle, 1980). It has also been shown, using the antigen bovine serum albumin, that *in-vitro* proteolytic digestion produces protein fragments which are tolerogenic *in vivo*, possibly via suppressor cell activation (Dosa *et al.*, 1979). Although we have not yet elucidated the nature of circulating immunoreactive ovalbumin after feeding, the results of our experiments are consistent with the hypothesis that tolerogenic forms of protein are produced *in vivo* after digestion and absorption of protein antigens in the gut, and that these are important in the induction of oral tolerance.

A striking finding in these experiments has been that transfer of serum from ovalbumin fed donors into naive recipients had no suppressive effect on subsequent systemic humoral immunity of recipients, but did lead to tolerance for CMI. We have found that these two limbs of the immune response differ in their susceptibility to tolerance induction by feeding different doses of ovalbumin, with a low dose of fed ovalbumin suppressing CMI but not antibody responses (Mowat *et al.*, 1982). These experiments confirm and extend our earlier suggestion that there is likely to be more than one regulatory factor involved in modulation of systemic immunity after the feeding of protein antigens.

In our previous experiments, the tolerance of systemic CMI after the feeding of ovalbumin was abrogated by cyclophosphamide, as was the CMI tolerance which occurred in recipients of ovalbumin-fed serum in the present report. The unequivocal observation that cyclophosphamide pretreatment of donors did not alter the tolerogenic properties of serum obtained after the feeding of ovalbumin is strong evidence to support our previous suggestion that the effects of cyclophosphamide on immune responses to fed ovalbumin are not due to associated changes in the intestinal epithelium. Rather we propose that the induction of oral tolerance for CMI in mice fed ovalbumin is due to a population of cyclophosphamide-sensitive suppressor T cells, which are activated by protein moieties generated from ovalbumin within the mucosa of the gastrointestinal tract. In the intact animal, dietary antigens will presumably interact primarily with the suppressor T cells which other workers have identified within gut-associated lymphoid tissues (Mattingly & Waksman, 1980; Richman *et al.*, 1978). Since parenteral administration of intestinally processed protein produced a state identical to oral tolerance, it is obviously not essential for the antigen meeting these cells to be reaching the gut-associated lymphoid tissues via the intestinal epithelium. Nevertheless, our results underline the close association between absorptive and immune functions of the intestine, and regulation of immune responses to dietary proteins.

ACKNOWLEDGMENTS

This work has been supported by a grant from the Medical Research Council. Dr Mowat was in receipt of the Allan Fellowship of the University of Edinburgh and Dr Strobel a Fellowship from the Deutsche Forschungsgemeinschaft. We acknowledge the skilled technical assistance of the staff of the Animal Unit, Western General Hospital.

REFERENCES

André C., Heremans J.F., Vaerman J. & Cambiaso C.L. (1975) A mechanism for the induction of immunological tolerance by antigen feeding: antigen–antibody complexes. *J. exp. Med.* **142,** 1509.

Asherson G.L., Perera M.A. & Thomas W.R. (1979) Contact sensitivity and the DNA response in mice to high and low doses of oxazalone, low dose unresponsiveness following painting and feeding and its prevention by pretreatment with cyclophosphamide. *Immunology*, **36,** 449.

Dosa S., Pesce A.J., Ford D.J., Muckerheide A. & Michael J.G. (1979) Immunological properties of peptic fragments of bovine serum albumin. *Immunology*, **38,** 509.

Kagnoff M.F. (1978) Effects of antigen feeding on intestinal and systemic immune responses. III. Antigen-specific serum-mediated suppression of humoral antibody responses after antigen-feeding. *Cell. Immunol.* **40,** 186.

Kagnoff M.F. (1980) Effects of antigen feeding on intestinal and systemic immune responses. IV. Similarity between the suppressor factor in mice after erythrocyte-lysate injection and erythrocyte feeding. *Gastroenterology*, **79,** 54.

Mattingly J.A. & Waksman B.H. (1980) Immunologic suppression after oral administration of antigen. II. Antigen-specific helper and suppressor factors produced by spleen cells of rats fed sheep erythrocytes. *J. Immunol.* **125,** 1044.

Miller S.D. & Hanson D.G. (1979) Inhibition of specific immune responses by feeding protein antigens. IV. Evidence for tolerance and specific active suppression of cell mediated immune responses to ovalbumin. *J. Immunol.* **123,** 2344.

MOWAT A.M. & FERGUSON A. (1981) Hypersensitivity in the small intestinal mucosa. V. Induction of cell-mediated immunity to a dietary antigen. *Clin. exp. Immunol.* **43,** 574.

MOWAT A.MCI., STROBEL S., DRUMMOND H.E. & FERGUSON A. (1982) Immunological responses to fed protein antigens in mice. I. Reversal of oral tolerance to ovalbumin by cyclophosphamide. *Immunology*, **45,** 105.

NGAN J. & KIND L.S. (1978) Suppressor T cells for IgE and IgG in Peyer's patches of mice made tolerant by the oral administration of ovalbumin. *J. Immunol.* **120,** 861.

PARKS D.E. & WEIGLE W.O. (1980) Maintenance of immunologic unresponsiveness to human γ-globulin: evidence for irreversible inactivation in B lymphocytes. *J. Immunol.* **124,** 1230.

RICHMAN L.K., CHILLER J.M., BROWN W.R., HANSON D.G. & VAZ N.M. (1978) Enterically induced immunologic tolerance. I. Induction of suppressor T lymphocytes by intra-gastric administration of soluble proteins. *J. Immunol.* **121,** 2429.

RÖLLINGHOFF M., STARZINSKI-POWITZ A., PFIZENMAIER K. & WAGNER H. (1977) Cyclophosphamide sensitive T lymphocytes suppress the *in vivo* generation of antigen specific cytotoxic T lymphocytes. *J. exp. Med.* **145,** 455.

SCHWARTZ A., ASKENASE P.W. & GERSHON R.K. (1978) Regulation of delayed type hypersensitivity reactions by cyclophosphamide-sensitive T cells. *J. Immunol.* **121,** 1573.

SWARBRICK E.T. (1979) Antigen handling by the gut. *MD Thesis.* Univ. of London.

VIVES J., PARKS D.E. & WEIGLE W.O. (1980) Immunologic unresponsiveness after gastric administration of human γ-globulin: antigen requirements and cellular parameters. *J. Immunol.* **125,** 1811.

WELLS H.G. & OSBORNE T.B. (1911) The biological reactions of the vegetable proteins. I. Anaphylaxis. *J. infect. Dis.* **8,** 66.

Immunology 1982 **45** 105

Immunological responses to fed protein antigens in mice. I. Reversal of oral tolerance to ovalbumin by cyclophosphamide

A. McI. MOWAT, S. STROBEL, HAZEL E. DRUMMOND & ANNE FERGUSON *Gastro-Intestinal Unit, University of Edinburgh and Western General Hospital, Edinbugh*

Accepted for publication 30 June 1981

Summary. Feeding ovalbumin over a wide range of doses is known to reduce subsequent systemic immune responses to parenteral immunization. In the present study, we have fed mice 2 mg and 25 mg ovalbumin (OVA) 2 weeks before systemic immunization and followed the resulting humoral antibody and cell-mediated immune (CMI) responses. The results indicate that while 25 mg OVA will reduce subsequent IgM, IgG and CMI responses to OVA, feeding 2 mg OVA will only suppress CMI responses and to a lesser extent the IgM response. Furthermore, the tolerant state induced by feeding 25 mg OVA was only partially prevented by 100 mg/kg cyclophosphamide (CY) while the suppressed CMI after feeding 2 mg OVA was completely blocked by CY pretreatment. These findings suggest that the humoral and cell-mediated limbs of the immune response may be controlled by different regulatory systems after feeding antigen, and that activation of these systems is dependent on the dose of oral antigen used. In addition, the results are in agreement with our previous finding that CY pretreatment will allow the development of CMI in the gut and gut-associated lymphoid tissue (GALT) after oral OVA and suggest that this phenomenon is related to breakdown of oral tolerance induction.

Abbreviations: GALT, gut-associated lymphoid tissues; CMI, cell-mediated immunity; OVA, ovalbumin; SRBC, sheep red blood cells; CY, cyclophosphamide; DTH, delayed-type hypersensitivity; FCA, Freund's complete adjuvant; HSA, human serum albumin; PBS, phosphate-buffered saline.

* Present address and correspondence: Dr A. McIntosh Mowat, Department of Bacteriology and Immunology, Western Infirmary, Glasgow.

0019-2805/82/0100-0105$02.00
© 1982 Blackwell Scientific Publications

INTRODUCTION

After an initial exposure to a protein antigen by the oral route, specific secretory antibodies of the IgA class are produced in the gut mucosa (Crabbé, Nash, Bazin, Eyssen & Heremans, 1969; Heremans, 1974). On the other hand, it is also well documented that feeding antigens leads to a state of specific unresponsiveness when the same antigen is met by a parenteral route (Chase, 1946; Thomas & Parrott, 1974; André, Heremans, Vaerman & Cambiaso, 1975; Hanson, Vaz, Maia, Hornbrook, Lynch & Roy, 1977; Swarbrick, Stokes & Soothill, 1979). The mechanisms responsible for this tolerant state remain controversial and may include immune suppression mediated by antibody (Kagnoff, 1978a; Kagnoff, 1980), immune complexes (André *et al.*, 1975) or induction of specific suppressor T cells in the GALT (Mattingly & Waksman, 1978; Ngan & Kind, 1978; Miller & Hanson, 1979). In particular, cellular suppressor mechanisms appear to be responsible for the unresponsiveness of CMI responses after feeding OVA (Miller & Hanson, 1979; Challacombe & Tomasi, 1980; Titus & Chiller,

1981, SRBC (Kagnoff, 1978b) and contact sensitizing agents (Asherson, Zembala, Perera, Mayhew & Thomas, 1977). We have previously argued that an important gut-associated suppressor cell system exists to prevent the induction of local immune responses in the gut and gut-associated lymphoid tissue (GALT) in addition to producing systemic tolerance in response to oral antigen. Abrogation or modulation of this homeostatic system may lead to the development of potentially harmful cell-mediated immunity (CMI) in the mucosa and GALT in response to dietary antigens and may be the mechanism underlying food allergic disease (Mowat & Ferguson, 1981).

In the mouse, suppressor T cells are sensitive to certain doses of cyclophosphamide (Askenase, Hayden & Gershon, 1975; Ferguson & Simmons, 1978; Gill & Liew, 1978) and so CY may be expected to interfere with the generation of 'normal' suppressor cells following oral antigen. In this case, it should be possible to detect the induction of CMI in the gut and its lymphoid tissues as well as to block the induction of systemic tolerance after antigen feeding. We have already shown that pretreatment with cyclophosphamide (CY) will indeed allow the induction of local CMI in the mucosa and GALT after oral sensitization and challenge with OVA (Mowat & Ferguson, 1981). The present experiments were designed to investigate whether a similar regime of CY treatment might also prevent the state of oral tolerance induced by a single feed of OVA.

Furthermore, we have examined both the humoral antibody response and CMI response in OVA fed mice, since it is possible that the two limbs of the immune response may be dissociated in their susceptibility to oral tolerance induction. Thus parallel experiments were performed using two different doses of oral protein as the tolerizing procedure to investigate their effects on the subsequent immune responses.

MATERIALS AND METHODS

Animals

Male and female BALB/c mice were first used at 6–8 weeks of age. These were bred in the Animal Unit, Western General Hospital and maintained on an ovalbumin-free diet throughout life.

Protocol of experiments

This is shown in Fig. 1 and the following groups of animals were used in each experiment. Group 1 received H_2O intraperitoneally on day −2 and H_2O orally on day 1. Group 2 received H_2O intraperitoneally on day −2 and 2 mg or 25 mg OVA (Sigma Fraction V) orally on day 1 as a tolerizing procedure. Group 3 received CY intraperitoneally on day −2 and 2 mg or 25 mg OVA on day 1. All mice were immunized 14 days after feeding, bled weekly for antibody levels and tested for DTH three weeks after immunization. Where appropriate, results of Groups 2 and 3 are expressed as a percentage suppression of the control response, given by: suppression (%) = [(control value − value in tolerant or CY treated mice)/(control value)] × 100.

Immunization

To assess the antibody response, animals were immunized with 2 mg OVA in Freund's complete adjuvant (FCA, Bacto-H37Ra Difco Ltd) intraperitoneally and bled at weekly intervals. In preliminary experiments, this regime was found to be ineffective in producing CMI. Therefore, for assessment of DTH separate groups of mice were immunized with 100 μg OVA in 0·05 ml FCA intradermally into one rear footpad and tested for delayed hypersensitivity 3 weeks later.

Antigen specificity

To determine the specificity of the tolerance induced by feeding OVA, mice were immunized with either 2 mg HSA (Sigma Fraction V) in FCA intraperitoneally or 100 μg human serum albumin (HSA) in FCA intradermally 2 weeks after feeding 25 mg OVA.

Cyclophosphamide

Cyclophosphamide (Endoxana—WB Pharmaceuticals) was given 100 mg/kg in distilled water intraperitoneally. Control animals were injected with 0·2 ml distilled water only.

	Group	*Day −2*	*Day 1*	*Day 14*	
1	Control	H_2O i.p.	H_2O orally	2 mg OVA/FCA i.p.	Bled weekly for Ab (8 mice/group)
2	OVA Fed	H_2O i.p.	2 mg or 25 mg OVA orally	or	
3	CY/OVA Fed	100 mg/kg CY i.p.	2 mg or 25 mg OVA orally	100 μg OVA/FCA i.d.	Tested for systemic DTH day 35 (8 mice/group)

Figure 1. Protocol of experiments designed to investigate the effect of CY pretreatment on the induction of oral tolerance by 2 mg or 25 mg ovalbumin.

Bleeding of mice

Mice were bled routinely from the retro-orbital plexus under light ether anaesthesia. Approximately 200 μl blood was removed from each mouse into heparinized hematocrit tubes (Propper Ltd), the tubes centrifuged and the sera stored at $-20°$, after inactivation at 56° for 30 min.

Haemagglutination assay

Sera were tested for antibodies to OVA or HSA by passive haemagglutination of coated sheep red blood cells (SRBC). SRBC in Alsever's solution were washed three times in saline at room temperature and 200 μl of packed cells were then mixed with 1·4 ml saline and 200 μl of protein solution (15 mg/ml OVA or 1 mg/ml HSA in saline). Two millilitres of 0·01% chromic chloride (Analar-BDH Ltd) in saline at pH5 was added dropwise with continuous agitation and the mixture allowed to stand at room temperature for 10 min. After the reaction had been stopped by addition of PBS pH 7·2, the coated cells were washed twice in PBS and finally resuspended at 1%.

All sera were absorbed with 10% SRBC before use. Serum (25 μl) was doubly diluted in round-bottomed, microtitre plates (Titertek Ltd) and 25 μl of coated SRBC added to each well. The plates were allowed to settle for 90 min at room temperature and the titre taken as the last dilution to show complete agglutination. All sera were tested with or without the presence of 25 μl 0·1 M 2-mercaptoethanol (Sigma Ltd) per well to obtain IgM and IgG antibody levels for each serum.

Antibody titres were converted to $\log_{10}$ units for statistical analysis.

Footpad testing for delayed-type hypersensitivity

Three weeks after immunization, mice were tested for delayed-type hypersensitivity (DTH) by the increment in footpad thickness 24 and 48 hr after an intradermal injection of 100 μg of the appropriate antigen (OVA or HSA) in 0·05 ml H_2O. Footpad thickness was measured by skinfold calipers (Carbobronze) immediately before and 24 and 48 hr after injection of antigen, and expressed in millimetres. Control animals were injected with 0·05 ml H_2O only, to assess the non-specific response.

Statistics

Groups of results ± standard errors were compared by Student's t test.

RESULTS

Effects of OVA prefeeding on serum antibody responses to parenteral OVA

IgM levels

Figures 2 and 3 show the mercaptoethanol-sensitive antibody responses in mice immunized intraperi-

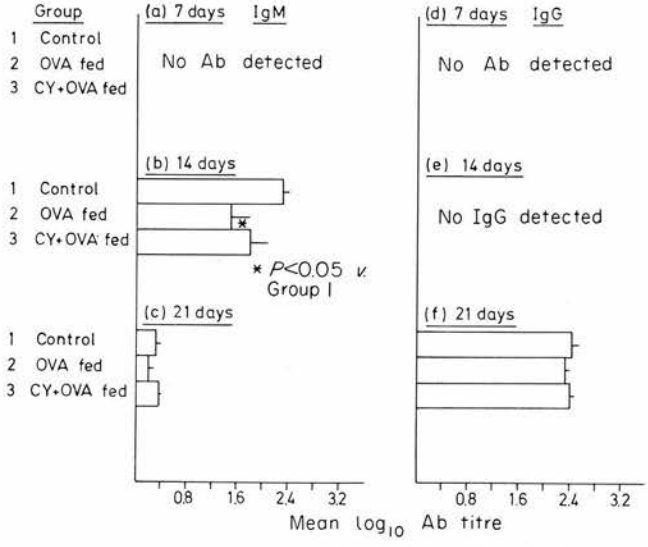


Figure 2. Serum haemagglutinating antibody responses (IgM and IgG) 1, 2 and 3 weeks after immunization with 2 mg OVA in FCA intraperitoneally in mice fed 2 mg OVA 14 days before immunization.

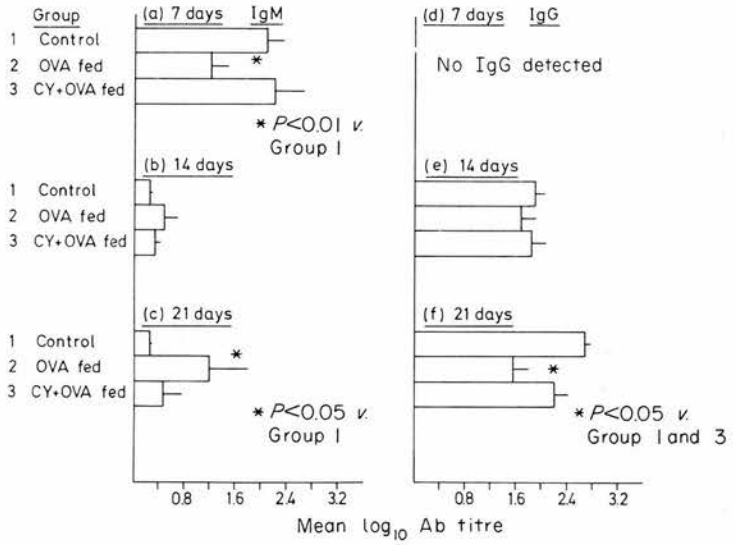


Figure 3. Serum haemagglutinating antibody responses (IgM and IgG) 1, 2 and 3 weeks after immunization with 2 mg OVA in FCA intraperitoneally in mice fed 25 mg OVA 14 days before immunization. Bars represent mean $\log_{10}$ titre + 1 standard error.

toneally with 2 mg OVA in FCA, 2 weeks after feeding 2 mg or 25 mg OVA, respectively. The peak of the IgM response occurred 2 weeks after immunization in animals fed 2 mg OVA and at 1 week in mice fed 25 mg OVA, at which times virtually all antibody was of the IgM class. It is seen that at these times, both doses of oral antigen have suppressed the subsequent IgM response compared with controls, with 93% suppression ($P < 0{\cdot}01$) in 25 mg OVA fed animals (Fig. 3a) and 60% suppression in 2 mg OVA fed mice ($P < 0{\cdot}05$, Fig 2a). The groups of mice pretreated with CY, before OVA feeding, have IgM levels which are identical to controls after feeding 25 mg OVA and are not significantly different from controls in the 2 mg OVA fed group (36% suppression).

IgG levels

In contrast to the results with the IgM responses in OVA fed mice where both doses of OVA suppressed the response, there was a marked discordance in the effect of 2 mg or 25 mg OVA on subsequent IgG responses (Figs 2 and 3). Mercaptoethanol-resistant antibody accounted for the majority of the antibody 3 weeks after immunization and it can be seen that at this time, feeding 25 mg OVA has markedly suppressed the IgG response of fed mice compared with controls (93% suppression $P < 0{\cdot}005$) (Fig. 3f). Mice fed 2 mg OVA on the other hand have IgG responses which are identical to controls. CY pretreatment of mice fed 25 mg OVA returns their IgG response towards normal (77% suppression) although this is not significantly different from either tolerant or control animals. CY pretreated, 2 mg OVA fed mice, have IgG levels which are the same as both other groups in this experiment.

Time course of antibody responses in mice fed 25 mg OVA

It was of interest to know if the maturation of the antibody response was also altered in the mice rendered tolerant by oral protein. In the three groups of mice fed 25 mg OVA, serum IgM and IgG levels could be followed from 1–3 weeks after immunization and these can be seen in Fig. 3a–e. All groups show the characteristic early peak of IgM production at one week (Fig. 3a) with higher levels of IgG later in the experiment (Fig. 3d–f). However, it is notable that after the marked suppression of IgM levels early in the response of the OVA fed mice, there is in addition a late rise in IgM antibody in these animals (Fig. 3c). This secondary rise in IgM 3 weeks after immunization is mirrored by a decline in IgG at this time (Fig. 3f) and is in marked contrast to the responses of control animals in whom IgM levels are very low by then. CY-pretreated mice, after an early IgM response similar to controls (Fig. 3a) have at later times a

pattern of IgG and IgM responses which is midway between the control and tolerant animals (Fig. 3c and 3f). When the results at 2 weeks are compared, the pattern of this response in the three groups is already emerging and is clearly associated with the switch from IgM to IgG production in the controls (Fig. 3b and 3e).

Thus, the results in Figs 2 and 3 indicate that the IgM responses of mice may be tolerized by even low doses of oral protein while larger amounts are required to achieve suppression of the IgG response. Also the tolerance of IgM responses appears more susceptible to abrogation by CY pretreatment. Variation between individual groups at each time in this experiment was quite large; however, the overall trend of the results in Fig. 3 indicates that the larger dose of oral OVA interferes with the maturation of the primary antibody response as well as suppressing absolute levels of antibody.

DTH responses in OVA fed mice

Figure 4 shows the DTH responses in mice fed 2 or 25 mg OVA, assessed 3 weeks after immunization by measuring the specific increase in footpad thickness 24 hr after an intradermal injection of 100 μg OVA in H_2O. The results in this figure illustrate that both doses of oral OVA will suppress a subsequent DTH response, with 88% suppression in 25 mg OVA fed mice ($P=0{\cdot}05$) and 64% suppression in the mice fed 2 mg OVA ($P=0{\cdot}05$) compared to controls. CY given to mice fed 25 mg OVA returns their systemic DTH responses to a value which is midway between the control and tolerant mice and which is not significantly different from either. It is important to note, however, that the mice receiving CY before feeding 2 mg OVA have completely normal DTH responses, and show no residual tolerance. Similar results were obtained 48 hr after testing (Fig. 4), confirming the DTH nature of the reactions measured, and the consistency of the tolerant state. Thus, feeding 25 mg OVA suppresses the humoral IgM, IgG and CMI responses while 2 mg OVA orally is effective only in reducing CMI and to a lesser extent the IgM antibody response. Furthermore, while both doses of oral OVA used produced significant suppression of subsequent DTH responses, the tolerance after feeding 25 mg OVA is only partially susceptible to CY pretreatment. Tolerance found after 2 mg OVA orally on the other hand is completely abrogated by CY.

Antigen specificity of oral tolerance induced by feeding OVA

To confirm that the state of tolerance induced by feeding OVA was specific to the fed protein, mice were

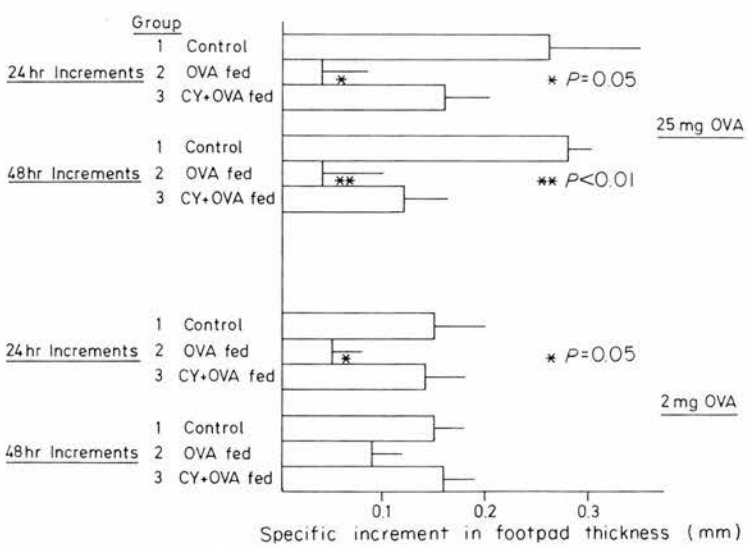


Figure 4. Systemic delayed hypersensitivity responses 3 weeks after immunization with 100 μg OVA in FCA intradermally in mice fed 2 mg or 25 mg OVA 14 days before immunization. Bars represent mean specific increments in footpad thickness (mm) 24 and 48 hr after 100 μg OVA in saline + 1 standard error.

also immunized with 2 mg HSA in FCA 2 weeks after feeding 25 mg OVA. Figure 5 shows the haemagglutinating anti-HSA responses 1 week and 3 weeks after immunization, and the OVA fed mice exhibit antibody responses to the unrelated protein which are the same as those of the H_2O fed controls.

Similarly, Figure 6 indicates that 3 weeks after intradermal immunization with 100 μg HSA in FCA, mice fed 25 mg OVA have normal DTH responses to HSA, assessed as the specific increase in footpad thickness 24 hr after 100 μg HSA in H_2O intradermally.

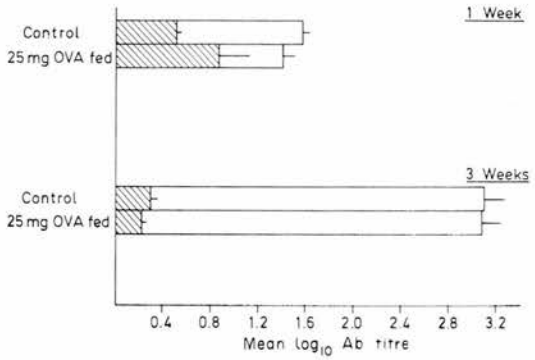


Figure 5. Serum haemagglutinating anti-HSA responses (IgM and IgG) 1 and 3 weeks after immunization with 2 mg HSA in FCA intraperitoneally in mice fed 25 mg OVA 14 days before immunization. Bars represent mean $\log_{10}$ titre +1 standard error. IgG (□) and IgM (▨) 3 weeks after immunization.

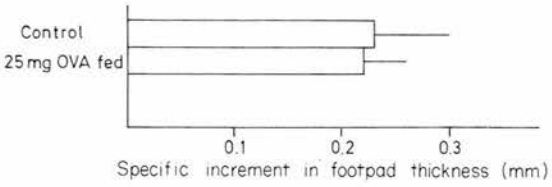


Figure 6. Systemic delayed hypersensitivity response 3 weeks after immunization with 100 μg HSA in FCA intradermally in mice fed 25 mg OVA 14 days before immunization. Bars represent mean specific increments in foodpad thickness 24 hr after 100 μg HSA in saline +1 standard error.

DISCUSSION

The results presented here confirm that when the first encounter with a protein antigen is by the oral route, a range of different effects on the systemic immune response may be observed. Thus, we have shown that depending on the dose of oral protein used, both humoral and cell-mediated immunity may be reduced; or alternatively, CMI alone may be reliably suppressed. In addition, our study indicates that unresponsiveness of the systemic antibody and CMI responses also differs in its susceptibility to abrogation by CY pretreatment.

A number of different experimental schedules have been used in the investigation of oral tolerance to protein antigens, and it is therefore necessary to emphasize that our results on the induction of oral tolerance to OVA are compatible with those of previous workers (Hanson *et al.*, 1977; Swarbrick *et al.*, 1979; Miller & Hanson, 1979; Challacombe & Tomasi, 1980; Titus & Chiller, 1981; Hanson, Vaz, Rawlings & Lynch, 1979; Vaz, Maia, Hanson & Lynch, 1977; Richman, Chiller, Brown, Hanson & Vaz, 1978). Thus we have used a single dose of oral OVA known to induce oral tolerance (25 mg) and a smaller dose (2 mg) which is nearer the lower limit of tolerogenicity (Hanson *et al.*, 1977; Challacombe & Tomasi, 1980; Vaz *et al.*, 1977). Similarly, although the optimum interval between feeding and immunization with OVA for the demonstration of systemic tolerance has been shown to be 7 days, similar unresponsiveness is found when a 14 day interval is employed as here (Challacombe & Tomasi, 1980; Vaz *et al.*, 1977). In the present study, it was hoped that differences in the susceptibility of the various components of the systemic immune response to oral tolerance might be observed. This was studied partly by the use of the high and low doses of oral OVA and by investigating the role of suppressor cells in the resulting states of unresponsiveness. Thus we have employed a dose of CY within the range known to inhibit some types of suppressor cells in the mouse when given shortly before the first encounter with antigen.

In addition to the discrepancy between the susceptibility of the antibody response and CMI response to reduction by prior feeding, it is apparent that differences exist within the humoral response itself. Although it has been reported that feeding OVA inhibits subsequent total serum antibody responses (Hanson *et al.*, 1977; Ngan & Kind, 1978; Hanson *et al.*, 1979) and serum IgE responses (Ngan & Kind, 1978; Vaz *et al.*, 1977), there is evidence to suggest that IgE may be more readily tolerized than IgG responses (Ngan & Kind, 1978). No such evidence exists at present on the relative susceptibilities of the IgM and IgG phases of the humoral response. In the present

study, we have shown that while both 2 mg OVA and 25 mg OVA feeding suppressed the subsequent peak IgM response to OVA (60% and 93% suppression, respectively), only the higher dose was effective in reducing IgG levels (98% suppression). Furthermore, mice receiving 2 mg OVA subsequently developed normal IgG levels despite a previously reduced IgM response, and it is clear that IgM production may be more readily tolerized by oral antigen. In support of this observation Kagnoff has reported that IgM plaque-forming cells are more susceptible to modulation by repeated feeding of SRBC (Kagnoff, 1978a).

Similarly, while systemic CMI responses are readily tolerized by OVA feeding (Miller & Hanson, 1979; Challacombe & Tomasi 1980), our study also indicates a dissociation between CMI and the IgG antibody response in their susceptibility to oral tolerance. Thus, CMI was markedly reduced by both doses of oral OVA, despite normal IgG levels in mice fed 2 mg OVA.

In the context of the different effects of oral OVA on systemic immune responses, the consequences of CY pretreatment in OVA fed mice are also relevant. In mice fed 25 mg OVA, similar degrees of suppression of IgM, IgG amd CMI responses were observed in each case and CY pretreatment returned these responses to a level midway between control and tolerant mice. A different pattern was seen in the mice fed 2 mg OVA however. In this case, the markedly suppressed CMI responses were returned to control levels by CY pretreatment. The reduced IgM responses however were not completely restored to normal by this treatment.

The induction of tolerance by parenteral antigen has been shown to have discordant effects on subsequent CMI and antibody responses (Neveu & Borduas, 1974; Silver & Benacerraf, 1974) and may involve suppressor cells (Basten, Miller, Sprent & Cheers, 1974), depletion of helper cells (Endres & Grey, 1980; Parks & Weigle, 1980) and B-cell inhibition (Parks & Weigle, 1980). It has been suggested previously that oral tolerance may have multiple control mechanisms (Titus & Chiller, 1981; Hanson *et al.*, 1979) and we propose that our findings support a system of oral tolerance dependent on two or more 'suppressor' mechanisms activated by protein feeding. These may include induction of suppressor cells, functional deletion of helper T cells and B-cell inhibition, their activation being dependent on the dose of antigen presented to the GALT.

While both suppressor cells or their products (Mattingly & Waksman, 1978; Mattingly & Waksman, 1980; Mattingly, Kaplan & Janeway, 1980) and serum factors (André *et al.*, 1975; Kagnoff, 1978a; Kagnoff, 1980) have been reported in the unresponsive state following feeding of particulate antigens, it is likely that suppressor T cells induced in the spleen and GALT are responsible for oral tolerance to protein antigens (Ngan & Kind, 1978; Miller & Hanson, 1979; Titus & Chiller, 1981; Richman *et al.*, 1978a). In particular, suppressor T cells are important in the reduction of CMI responses after feeding OVA (Miller & Hanson, 1979; Challacombe & Tomasi, 1980; Titus & Chiller, 1981). Furthermore the induction of tolerance by feeding contact-sensitizing agents to guinea-pigs is related to the appearance of suppressor cells (Asherson *et al.*, 1977) and is abrogated by CY (Polak, Geleick & Turk, 1975). Thus the tolerance of CMI response after a low dose of OVA which was fully sensitive to CY in our experiments suggests that a suppressor cell mechanism is entirely responsible for the tolerance of CMI responses after small doses of oral protein.

Although there is ample evidence to support the view that our regime of CY pretreatment is acting by its effect on suppressor lymphocytes, it is important to consider the effects that this drug may have on the ability of the gut to process proteins. However, this dose of CY has minimal effects on the integrity of the gut mucosa (Ecknauer & Löhrs, 1976; Hartwich, Weisshaar & Domschke, 1978), and we have found recently that the uptake of immunologically active OVA by the gut is not altered by CY pretreatment (Mowat, Strobel, Drummond, Pickering & Ferguson, unpublished observation). Further support for an immunological effect of CY has come from work showing that the development of suppressor T cells induced by OVA feeding to mice is inhibited by 100 mg/kg CY (D.G. Hanson, personal communication). It is unlikely therefore that intestinal damage can account for the results presented here.

The tolerance of antibody and CMI responses induced by feeding 25 mg OVA was however only partially reversible by CY pretreatment. This may reflect the induction by larger doses of oral protein of tolerogenic mechanisms additional to suppressor cells. Thus, feeding OVA to mice has been shown to induce defective helper T-cell function in addition to the simultaneous activation of suppressor T cells (Titus & Chiller, 1981). A defect in T-helper activity may therefore be produced in response to larger doses of oral protein. A defect of this nature is consistent with

the finding of an apparent disturbance in the switch from IgM to IgG synthesis in animals tolerized with 25 mg OVA (Fig. 3). A similar defect in an accessory T cell required for DTH expression may also account for the partial CY sensitivity of the CMI tolerance after feeding 25 mg OVA.

A further level of control may be responsible for the peculiar sensitivity of IgM production to oral tolerance. It is possible that a direct transient, inhibitory effect on B cells may be manifest as a defect in IgM synthesis. Indeed, although in certain circumstances B-cell priming may occur (Titus & Chiller, 1981), a defect of this type has been described after feeding proteins (Vives, Parks & Weigle, 1980). In the presence of helper T cells this may be overcome and a mature antibody response eventually mounted as seen in the 2 mg OVA fed mice. After feeding 25 mg OVA, functional deletion of helper T cells would not allow a full IgG response to occur, and if B-cell recovery were to take place, a delayed IgM response would ensue, as we have observed.

In conclusion, the results described here support the presence of several regulatory mechanisms governing systemic immune responses after feeding protein antigens. In particular, systemic CMI responses are more easily modulated by antigen feeding and a CY-sensitive suppressor mechanism may be responsible for this phenomenon. These findings concur with our previous report that a similar regime of CY pretreatment and OVA feeding allows the development of CMI in the GALT and intestinal mucosa (Mowat & Ferguson, 1981). Thus, mice rendered deficient in suppressor cells by CY show a loss of tolerance for CMI in addition to the induction of local CMI in the gut and its lymphoid tissues. These findings are consistent with the hypothesis that a homeostatically important suppressor cell system in the GALT normally protects the animal (and its gut) against deleterious CMI reactions to dietary antigen (Mowat & Ferguson, 1981).

ACKNOWLEDGMENTS

The authors would like to thank the staff of the Animal Unit, Western General Hospital, and in particular Miss June Swinton for their assistance with the animals used in these experiments. We also thank Mrs Doreen Orr and Mrs Alison Hardy who have prepared the manuscript.

Dr A. Mowat was in receipt of the Allan Fellowship from the University of Edinburgh. Dr S. Strobel is supported by Research Fellowship STR 210/1 from the Deutsche Forschungsgemeinschaft.

REFERENCES

André C., Heremans J.F., Vaerman J. & Cambiaso C.L. (1975) A mechanism for the induction of immunological tolerance by antigen feeding: antigen–antibody complexes. *J. exp. Med.* **142,** 1509.

Asherson G.L., Zembala M., Perera M.A.C.C., Mayhew B. & Thomas W.R. (1977) Production of immunity and unresponsiveness in the mouse by feeding contact sensitising agents and the role of suppressor cells in the Peyer's Patches, mesenteric lymph nodes and other lymphoid tissues. *Cell Immunol.* **33,** 145.

Askenase P.W., Hayden B.J. & Gershon R.K. (1975) Augmentation of delayed-type hypersensitivity by doses of cyclophosphamide which do not affect antibody responses. *J. exp. Med.* **141,** 697.

Basten A., Miller J.F.A.P., Sprent J. & Cheers C. (1974) Cell-to-cell interaction in the immune response X. T cell dependent suppression in tolerant mice. *J. exp. Med.* **140,** 199.

Challacombe S.J. & Tomasi T.B. (1980) Systemic tolerance and secretory immunity after oral immunisation. *J. exp. Med.* **152,** 1459.

Chase M.W. (1946) Inhibition of experimental drug allergy by prior feeding of the sensitising agent. *Proc. Soc. exp. Biol. Med. (N.Y.)*, **61,** 257.

Crabbé P.A., Nash D.R., Bazin H., Eyssen H. & Heremans J.F. (1969) Antibodies of the IgA type in intestinal plasma cells of germfree mice after oral or parenteral immunisation with ferritin. *J. exp. Med.* **130,** 723.

Ecknauer R. & Löhrs U. (1976) The effect of a single dose of cyclophosphamide on the jejunum of specified pathogen-free and germfree rats. *Digestion*, **14,** 269.

Endres R.O. & Grey H.M. (1980) Antigen recognition by T cells. II. Intravenous administration of native or denatured ovalbumin results in tolerance to both forms of antigen. *J. Immunol.* **125,** 1521.

Ferguson R.M. & Simmons R.L. (1978) Differential cyclophosphamide sensitivity of suppressor and cytotoxic cell precursors. *Transplantation*, **25,** 36.

Gill H. & Liew F.Y. (1978) Regulation of delayed-type hypersensitivity III. Effect of cyclophosphamide on the suppressor cells for delayed-type hypersensitivity to sheep erythrocytes in mice. *Europ. J. Immunol.* **8,** 172.

Hanson D.G., Vaz N.M., Maia L.C.S., Hornbrook M.M., Lynch J.M. & Roy C.A. (1977) Inhibition of specific immune responses by feeding protein antigens. *Int. Archs. Allergy appl. Immunol.* **55,** 526.

Hanson D.G., Vaz N.M., Rawlings L.A. & Lynch J.M. (1979) Inhibition of specific immune responses by feeding protein antigens II. Effects of prior passive and active immunisation. *J. Immunol.* **122,** 2261.

Hartwich Von G., Weisshaar K. & Domschke W. (1978) Intestinale Disaccharidasen der Ratte unter Cyclophosphamid-Behandlung. *Arzneim-Forsch.* **28,** 973.

HEREMANS J.F. (1974) Immunoglobulin A In: *The Antigens* (Ed. by M. Sela), p. 365. Academic Press, New York.

KAGNOFF M.F. (1978a) Effects of antigen feeding on intestinal and systemic immune responses III. Antigen-specific serum-mediated suppression of humoral antibody responses after antigen-feeding. *Cell Immunol.* **40,** 186.

KAGNOFF M.F (1978b) Effects of antigen feeding on intestinal and systemic immune responses II. Suppression of delayed-type hypersensitivity reactions. *J. Immunol.* **120,** 1509.

KAGNOFF M.F. (1980) Effects of antigen feeding on intestinal and systemic immune responses IV. Similarity between the suppressor factor in mice after erythrocyte-lysate injection and erythrocyte feeding. *Gastroenterology*, **79,** 54.

MATTINGLY J.A., KAPLAN J.M. & JANEWAY C.A. (1980) Two distinct antigen-specific suppressor factors induced by the oral administration of antigen. *J. exp. Med.* **152,** 445.

MATTINGLY J.A. & WAKSMAN B.H. (1978) Immunologic suppression after oral administration of antigen I. Specific suppressor cells formed in rat Peyer's patches after oral administration of sheep erythrocytes and their systemic migration. *J. Immunol.* **121,** 1878.

MATTINGLY J.A. & WAKSMAN B.H. (1980) Immunologic suppression after oral administration of antigen II. Antigen-specific helper and suppressor factors produced by spleen cells of rats fed sheep erythrocytes. *J. Immunol.* **125,** 1044.

MILLER S.D. & HANSON D.G. (1979) Inhibition of specific immune responses by feeding protein antigens IV. Evidence for tolerance and specific active suppression of cell mediated immune responses to ovalbumin. *J. Immunol.* **123,** 2344.

MOWAT A.M. & FERGUSON A. (1981) Hypersensitivity in the small intestinal mucosa V. Induction of cell-mediated immunity to a dietary antigen. *Clin. exp. Immunol.* **43,** 574.

NEVEU P.J., & BORDUAS A.G. (1974) Carrier function in immune deviation. *J. Immunol.* **112,** 1264.

NGAN J. & KIND L.S. (1978) Suppressor T cells for IgE and IgG in Peyer's patches of mice made tolerant by the oral administration of ovalbumin. *J. Immunol.* **120,** 861.

PARKS D.E. & WEIGLE W.O. (1980) Maintenance of immunologic unresponsiveness to human γ-globulin: Evidence for irreversible inactivation in B lymphocytes. *J. Immunol.* **124,** 1230.

POLAK L., GELEICK H. & TURK J.L. (1975) Reversal by cyclophosphamide of tolerance in contact sensitisation: tolerance induced by prior feeding with DNCB. *Immunology*, **28,** 939.

RICHMAN L.K., CHILLER J.M., BROWN W.R., HANSON D.G. & VAZ N.M. (1978) Enterically induced immunologic tolerance I. Induction of suppressor T lymphocytes by intra-gastric administration of soluble proteins. *J. Immunol.* **121,** 2429.

SILVER J. & BENACERRAF B. (1974) Dissociation of T cell helper function and delayed hypersensitivity. *J. Immunol.* **113,** 1872.

SWARBRICK E.T., STOKES C.R. & SOOTHILL J.F. (1979) Absorption of antigens after oral immunisation and the simultaneous induction of specific systemic tolerance. *Gut*, **20,** 121.

THOMAS H.C. & PARROTT D.M.V. (1974) The induction of tolerance to a soluble protein antigen by oral immunisation. *Immunology*, **27,** 631.

TITUS R.G. & CHILLER J.M. (1981) Orally induced tolerance: Definition at the cellular level. *Int. Archs. Allergy appl. Immunol.* **65,** 323.

VAZ N.M., MAIA L.C.S., HANSON D.G. & LYNCH J.M. (1977) Inhibition of homocytotropic antibody responses in adult inbred mice by previous feeding of the specific antigen. *J. Allergy clin. Immunol* **60,** 110.

VIVES J., PARKS D.E. & WEIGLE W.O. (1980) Immunologic unresponsiveness after gastric administration of human γ-globulin: antigen requirements and cellular parameters. *J. Immunol.* **125,** 1811.

Ann. N.Y. Acad Sci
1983, 409: 486-497

ABROGATION OF TOLERANCE TO FED ANTIGEN AND INDUCTION OF CELL-MEDIATED IMMUNITY IN THE GUT-ASSOCIATED LYMPHORETICULAR TISSUES*

Anne Ferguson, A. McI. Mowat,† and S. Strobel

Gastrointestinal Unit
University of Edinburgh
Edinburgh, Scotland

INTRODUCTION

There are many T-lymphocytes not only within the organized lymphoid tissues of the gastrointestinal tract, but also scattered within the mucosa as intraepithelial and lamina propria lymphocytes. Specific immunological responses to the feeding of protein antigen are multiple, and include a range of systemic humoral and cell-mediated immune responses, together with mucosal immunity (FIGURE 1). There is an increasing weight of evidence, from work in rodents, that the specific immunological responses to fed antigen are regulated by immunoregulatory T-cells in the organized lymphoid tissues of the gut. Until recently, our research program has concerned the effector limb of intestinal T-cell-mediated immunity (CMI). By using the animal models of allograft rejection of intestine, and graft-versus-host reaction, we have produced evidence that when a CMI reaction occurs in the small intestine mucosa, there is villous atrophy, crypt hyperplasia, and lymphocyte infiltrate—lesions associated with malabsorption.[1-5] Intestinal mucosal immune responses to food antigens are likely to be implicated in celiac disease and in other malabsorption syndromes with food protein intolerance.[6] Allergy to foods is an important component of atopic disease in infants.[7] In these and similar diseases, the primary pathology must be accepted as an abrogation of the normal, usually harmless immune response to dietary antigens, and so there is a need for the study of the mechanisms underlying induction of the various types of immune response to fed antigens, particularly induction of mucosal cell-mediated immunity.

Induction of systemic tolerance by feeding antigen is well documented,[8-11] although the mechanisms responsible remain controversial. There is, however, persuasive evidence that cellular suppressor mechanisms are related to systemic tolerance for CMI after the feeding of ovalbumin.[12-14] We therefore, empirically, decided to perform a series of experiments in which we would attempt to induce intestinal CMI to ovalbumin in mice that were orally immunized after pretreatment with cyclophosphamide (CY), because administration of CY in the dose 100 mg/kg enhances CMI reactions without an appreciable effect on antibody synthesis, by way of suppressor-cell inhibition.[15] These experiments also allowed us to develop an *in vitro* method for detection of CMI in the lymphoid tissues of

*This work has been supported by a grant from the Medical Research Council of the United Kingdom. Dr. A. Mowat was a recipient of the Allan Fellowship of the University of Edinburgh; Dr. S. Stobel was the recipient of a grant from the Deutsche Forschungsgemeinschaft, Str 210/2-3.

†Present address: Registrar in Bacteriology and Immunology, Department of Bacteriology and Immunology, Western Infirmary, Glasgow, Scotland.

the gut. The experimental details and results have been published elsewhere in full.[16-18]

Methods

Animals

BALB/c mice aged 6 to 10 weeks were used throughout. These mice were bred in the Animal Unit, Western General Hospital, Edinburgh, and maintained under conventional conditions on a pelleted diet that did not contain egg protein.

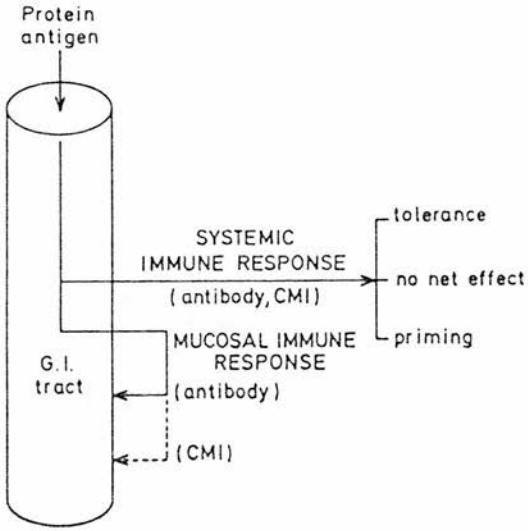


FIGURE 1. Diagrammatic representation of the range of systemic and mucosal immune responses to a fed protein antigen. On present evidence, there is a mucosal antibody response, but induction of mucosal CMI occurs only under experimental conditions or in disease.

Cyclophosphamide Treatment

Cyclophosphamide (Endoxana, WB Pharmaceuticals) was dissolved in saline and mice were given 100 mg/kg intraperitoneally two days before primary oral immunization with ovalbumin.

Oral Immunization

For primary immunization, mice were fed a single dose of either 2 mg or 25 mg ovalbumin (OVA) (Sigma Fraction V) in saline by intragastric tube. In some experiments a repeat feeding was performed in the following way. Twenty-eight days after the primary immunization, OVA, 2 mg/100 ml, was dissolved in the

animals' drinking water so that each mouse ingested approximately 0.1 mg OVA daily. This feeding was continued for 10 days.

Skin Testing for Delayed Hypersensitivity

The presence of systemic delayed type hypersensitivity (DTH) was assessed in the various groups of immunized mice by intradermal skin tests using either flank or footpad skin. Mice were injected with 100 μg OVA in 0.05 ml saline intradermally on both shaven flanks, and double skinfold thickness was measured with calipers immediately before and 24 hours after injection, results being expressed as mean increment in skin thickness in millimetres at 24 hours. In other experiments, mice were tested by the increment in footpad thickness 24 hours after an intradermal injection of 100 μg OVA in 0.05 ml water. Control animals were injected with 0.05 ml saline only to assess the nonspecific response.

Lymphocyte Migration Inhibition Test

Mesenteric lymph nodes (MLN) from three to four mice were removed, washed in RPMI 1640 medium (Flow Laboratories) and trimmed of surrounding material before being gently cut up and passed through a fine wire mesh filter. After one passage through a 25 gauge needle, the cells were washed three times in RPMI 1640 and finally adjusted to 100×10^6 cells/ml in RPMI 1640. The cells were supplemented with 2 mmol/l glutamine, 100 μg/ml penicillin G, and 100 μg/ml streptomycin and buffered with 10 mmol/l hepes buffer. A standard migration inhibition test was then performed using 10 μl capacity glass capillary tubes in macrophage migration inhibition test plates (Sterilin Ltd.), the wells being filled either with 0.45 ml supplemented RPMI 1640 medium alone or medium containing 0.1 mg/ml ovalbumin. Six to eight wells were used for each antigen dose or for control cultures. After incubation at 37° C for 20 hours, migration areas were measured using a drawing tube attached to a dissecting microscope. The migration index was calculated as the mean area of migration in wells containing antigen divided by the mean area of migration in control wells.

Small Intestinal Epithelial Cell Kinetics

For measurements of intestinal mucosal architecture and mitotic activity of the crypts of Lieberkühn, villus length, crypt length, and crypt cell-production rate were examined in groups of animals by a technique first described by Clarke,[19] and previously used by our group in experiments on allograft rejection of gut.[3] Briefly, on the day of killing, all animals were given 7.5 mg/kg colchicine (BDH Pharmaceuticals Ltd.) intraperitoneally (i.p.), to arrest mitosis in metaphase. Individual animals were then killed at intervals from 20 to 100 minutes thereafter. Pieces of jejunum, 10 cm from the pylorus, were removed and fixed in 75% ethanol/25% acetic acid for six hours, then stained with a modified Feulgen stain. Villus length and crypt length were expressed in microns (μm) and the crypt cell-production rate (CCPR) expressed as net accumulation of metaphases/crypt/hour.

Intraepithelial Lymphocyte Counts

Pieces of jejunum were fixed in 10% buffered formal saline, paraffin embedded, 5 μm sections cut and stained with hematoxylin and eosin. A differential count was made of cell types within the epithelium covering the villi,[20] and the intraepithelial lymphocyte (IEL) count was expressed as the number of IEL/100 epithelial cells.

Experiments and Results

Separate groups of animals were used for the measurements of lymph node cell migration, intestinal epithelial cell kinetics and IEL counts, studies of systemic immunization, and of systemic tolerance. Further groups of animals were used as positive controls having been systemically immunized with OVA in complete Freund's adjuvant; specificity controls were also performed using human serum albumin as an antigen.[17,21] Animals remained healthy throughout the experiments and the CY pretreated animals had no clinical side effects, weight gain being similar in all groups.

In general, four groups of animals were studied for each type of response. Untreated controls received water i.p. on day -2 and a water feed on day one. CY alone: animals received CY on day -2 and water or no further treatment thereafter. OVA alone: animals received water i.p. on day -2 and OVA feeding on day one. CY/OVA: animals received 100 mg/kg CY i.p. on day -2, and a feed of OVA on day one.

Systemic Immunity

The validity of intradermal skin testing as a measure of systemic DTH was confirmed by parenteral immunization of four mice that had received OVA in Freund's complete adjuvant and 21 days later were skin tested with 100 μg OVA (Figure 2). Similar skin testing was performed in orally immunized mice at 21 and 39 days after OVA feeding; no positive reactions were elicited in any of the orally immunized groups, including the CY/OVA treated animals (Figure 2).[18,21]

Systemic Tolerance

The presence or absence of tolerance induction was examined in the various experimental groups by immunizing all animals with 100 μg OVA in Freund's complete adjuvant intradermally, with DTH responses being measured 21 days later by a footpad swelling test. Results are summarized in Figure 3. Both doses of OVA used, 2 and 25 mg, suppressed a subsequent DTH response with 88% and 84% suppression ($p = 0.05$ in each case) in the fed mice when compared with water fed controls. The mice that were treated with CY before the feeding of 2 mg OVA had completely normal systemic DTH responses with no residual tolerance, and those mice CY pretreated, fed 25 mg OVA, had systemic DTH responses midway between control and tolerant animals, the value not being significantly different from either. Thus the tolerance for systemic DTH that was found after the feeding of OVA was abrogated by CY pretreatment.

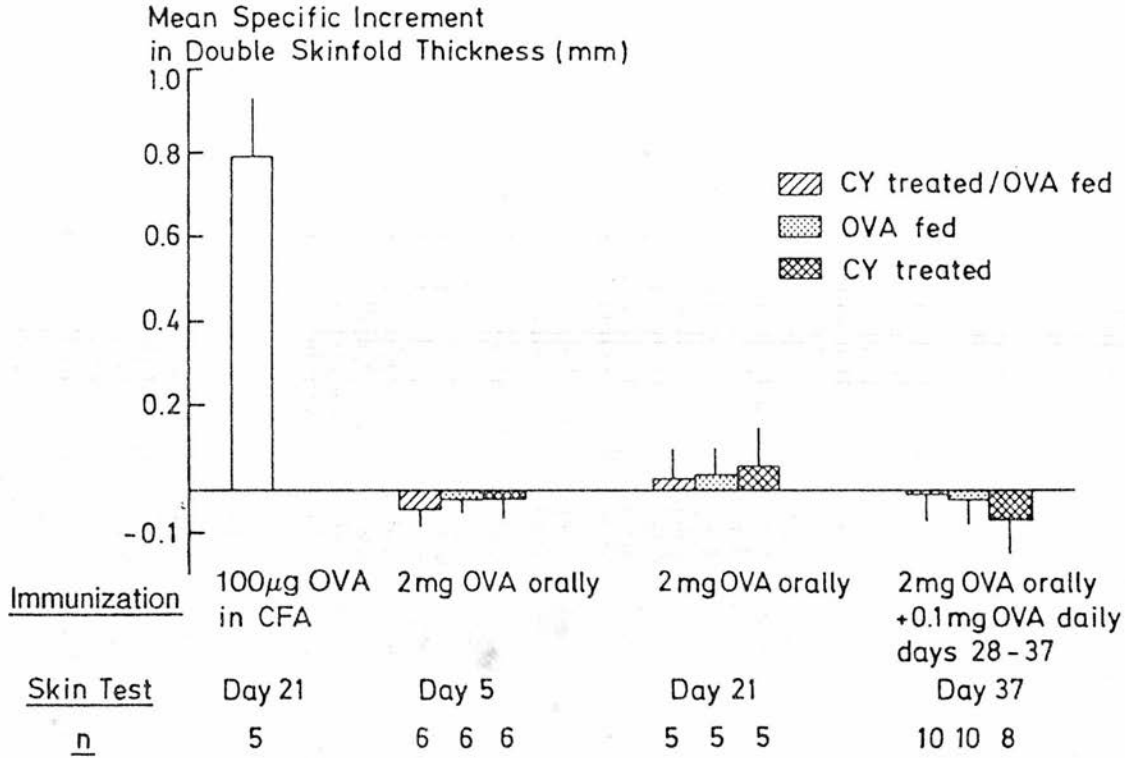


FIGURE 2. Systemic DTH responses, measured by skin test, after primary and secondary oral immunization with OVA in mice. A group of mice was also immunized with 100 μg OVA in complete Freund's adjuvant as positive controls. Results are expressed as mean specific increment in double skinfold thickness, 24 hours after 100 μg OVA in saline intradermally. Mean + 1 SD. Interval between immunization and skin test, and number of mice per group, are as shown.

Intestinal Cell Mediated Immunity—Migration Inhibition of Mesenteric Lymph Node Cells

Migration inhibition tests were performed with 0.1 mg/ml OVA and MLN cells taken at intervals from 1 to 22 days after oral immunization. Results are summarized in FIGURE 4. Animals fed OVA alone had no migration inhibition at any time, but animals given OVA preceded by CY had significant inhibition of migration at 24 hours after the OVA feed. A similar degree of migration inhibition persisted until 13 days after feeding. Two specificity controls were performed.[18] There was no migration inhibition of MLN in cells from OVA-immunized animals when the cells were incubated in the presence of human serum albumin, nor did OVA itself inhibit migration of MLN cells from unimmunized controls.

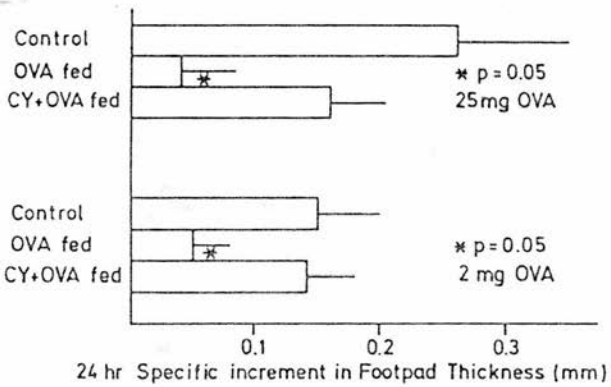


FIGURE 3. Effect of CY pretreatment on systemic tolerance induced by an OVA feed. Systemic DTH responses were measured by footpad swelling test three weeks after systemic immunization of all animals with 100 μg OVA in complete Freund's adjuvant. Bars represent mean specific increment in footpad thickness (millimetres) 24 hours after 100 μg OVA in saline, plus 1 SE. The three experimental groups are as shown. Prior OVA feeding, at both doses studied, induced systemic tolerance that was abrogated by CY pretreatment.

Intestinal Mucosal Architecture Changes after a Repeat Feed of Ovalbumin

Measurements of villus and crypt length, CCPR and IEL counts were made 10 days after OVA was fed again to the four experimental groups. Our previous work has shown that these two features can be used as indirect measures of the presence of a mucosal CMI reaction.[4,5] Although on conventional histology no abnormality of the mucosa was detected in any of the groups, objective measurements revealed minor but significant differences in the CY/OVA experimental mice. Results are illustrated in FIGURE 5. Villus length, crypt length, and CCPR were similar for the exclusively CY and OVA mice and untreated controls. CY/OVA animals, however, given a 10 day oral challenge with OVA, had significantly increased crypt length and CCPR compared with the other experimental groups. There were no significant differences between the groups with respect to villus length.

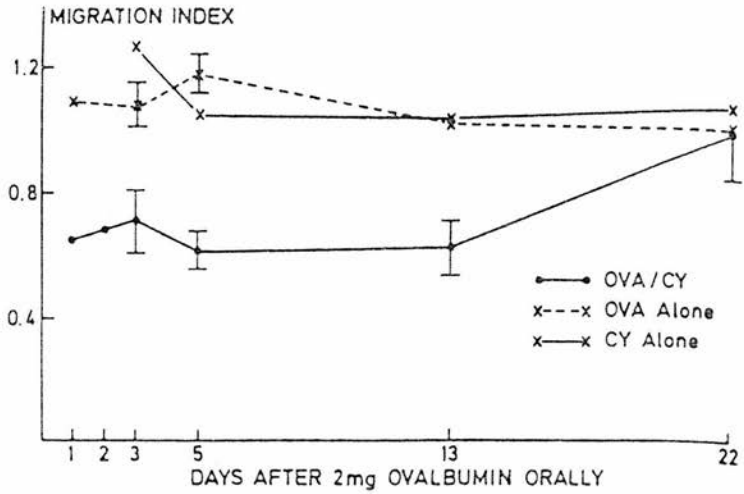


FIGURE 4. Development of migration inhibition in the MLN of mice fed OVA after CY pretreatment, and in mice fed exclusively OVA or given only CY. Where shown, bars represent mean ± 1 SD of three experiments (OVA/CY groups versus others, $p < 0.01$). Other results are for one experiment, and statistical analysis between groups was not possible.

Intraepithelial Lymphocyte Counts

As illustrated in FIGURE 6, IEL counts were significantly higher in the CY/OVA animals (mean 25.9) than in the other experimental and control groups (14.5, 15.9, and 14.1).

DISCUSSION

The experiments described in this paper have shown that under certain circumstances, oral immunization with the protein antigen OVA can induce a local CMI response in the gut-associated lymphoreticular tissues. Mice pretreated with 100 mg/kg CY before a feed of OVA had significant inhibition of migration in the presence of antigen and of MLN cells. They also had alterations in the structure of the intestinal mucosa after a repeat feeding of ovalbumin. These results suggest that CY pretreatment has induced intestinal cell-mediated immune responses.

The mechanism by which CY treatment has produced these effects in the gut is likely to be analogous to that which has been established in experiments on systemic immune responses—release of CMI reactions from suppressor control.[12-15] Thus we postulate that all of the effects observed in CY/OVA treated animals could be due to the effect of CY on T-suppressor cells. Two other possible actions of CY, however, should also be considered. The small intestine epithelium is one of the principal target organs for the cytotoxicity of many drugs, including CY, and so an increased permeability to proteins, or reduced enzyme content of cells of the intestinal mucosa may also be present. Alterations in the

antigen handling properties of the gastrointestinal tract may influence CMI responses by leading to absorption of an unusually large amount of antigen, or alterations in the immunochemical nature of absorbed antigens. The maximal effect on the intestinal epithelium, however, of the dose of CY used occurs at 12-24 hours after drug administration,[22] and in our experiments OVA was fed at 48 hours. In addition to this potential alteration in the villus epithelium, an effect of CY on the lymphoid tissues of the gut, including Peyer's patches and surface epithelium overlying Peyer's patches, also deserves consideration; however, we have no information on this effect at the moment.

Support for the hypothesis that in these experiments we have reversed a background of homeostatic suppression of gut-associated CMI responses, comes from the considerable body of work on regulation of systemic delayed type hypersensitivity. The concept has arisen that there is normally a considerable

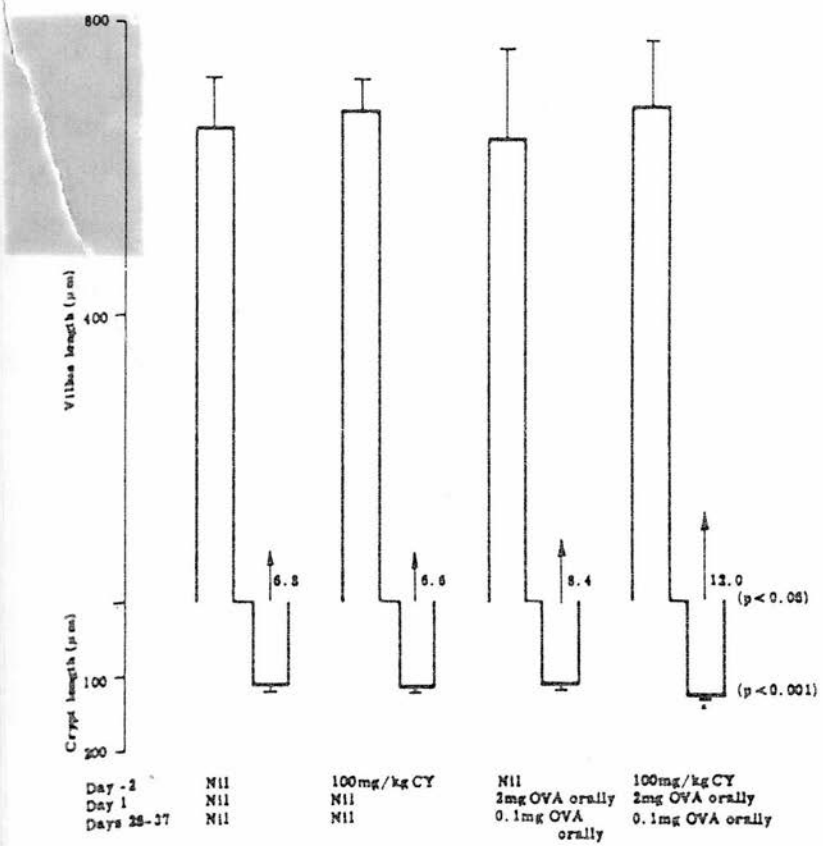


FIGURE 5. Mucosal architecture in jejunum of BALB/c mice treated as shown. Mucosal changes were measured after 10 days oral challenge with 0.1 mg OVA/day. Bars show means ± 1 SD for villus and crypt lengths, and arrows represent CCPR (six to eight mice per group).

degree of inhibitory control operating on the induction phase of DTH responses, and although we cannot draw any firm conclusions from the work described in this paper as to the nature of the postulated suppressor cells involved in regulating intestinal CMI, it is probably significant that T-suppressor cells for antibody production have been detected in Peyer's patches of orally immunized mice.[24,25] A similar system appears to exist for cell-mediated immune responses following oral immunization.[12] Future work on intestinal mucosal CMI responses and extension of this animal work into the clinical sphere, will require methods to detect and measure such responses. Our previous research on allograft rejection

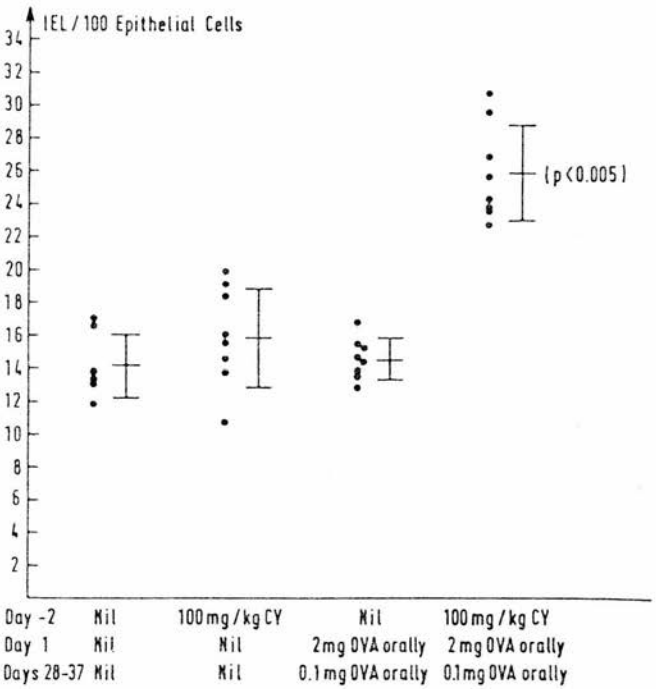


FIGURE 6. Intraepithelial lymphocyte counts in jejunum of BALB/c mice treated as shown. IEL counts measured after 10 days of oral challenge with 0.1 mg OVA/day. Results are expressed as mean ± 1 SD for each group of six to eight mice.

and graft-versus-host reaction identified the CCPR and IEL counts as reliable indices of mucosal CMI,[2-5] and these two parameters are sensitive, if not necessarily specific, indicators of a mucosal CMI reaction in experimental animals. These indices can now be supplemented by lymphocyte migration inhibition tests on MLN cells, and these tests should allow more detailed investigation of the effects on the intestinal mucosa and its lymphoid tissues of the manipulation of cell-mediated immune responses to fed antigens.

There is a growing body of evidence that says that mucosal T-cells represent a separate pool of lymphocytes from the systemic T-cell system, and have unique origins, routes of migration, and functions.[26] In theory, activation of the gut-

associated lymphoreticular tissues with subsequent migration and differentiation of gut-associated lymphocytes need not involve activation of systemic-immune responses. Our findings of enhanced CMI responses in the gut in the absence of systemic DTH is further evidence supporting the segregation of mucosal and systemic T-cell populations.

Acknowledgment

We acknowledge the assistance of the staff of the Animal Unit, Western General Hospital, Edinburgh.

References

1. Ferguson, A. & D. M. V. Parrott. 1973. Histopathology and time-course of rejection of allografts of mouse small intestine. Transplantation **15:** 546–554.
2. MacDonald, T. T. & A. Ferguson. 1976. Hypersensitivity reactions in the small intestine. 2. Effects of allograft rejection on mucosal architecture and lymphoid cell infiltrate. Gut **17:** 81–91.
3. MacDonald, T. T. & A. Ferguson. 1977. Hypersensitivity reactions in the small intestine. 3. The effects of allograft rejection and of graft-versus-host disease on epithelial cell kinetics. Cell Tissue Kinet. **10:** 301–312.
4. Mowat, A. McI. & A. Ferguson. 1981. Hypersensitivity reactions in the small intestine. 6. Pathogenesis of the graft-versus-host reaction in the small intestinal mucosa of the mouse. Transplantation **32:** 238–243.
5. Mowat, A. McI. & A. Ferguson. 1982. Intraepithelial lymphocyte count and crypt hyperplasia measure the mucosal component of the graft-versus-host reaction in mouse small intestine. Gastroenterology. **83:** 417–423.
6. Ferguson, A. & A. McI. Mowat. 1980. Immunological mechanisms in the small intestine. *In* Recent advances in gastrointestinal pathology. R. Wright, Ed.: 93–103. W. B. Saunders. Eastbourne, England.
7. Bock, S. A., W. Y. Lee, K. K. Remigio & C. D. May. 1978. Studies of hypersensitivity reactions for foods in infants and children. J. Allergy Clin. Immunol. **62:** 327–334.
8. Thomas, H. C. & D. M. V. Parrott. 1974. The induction of tolerance to a soluble protein antigen by oral immunisation. Immunology **27:** 631–639.
9. Andre, C., J. F. Heremans, J. Vaerman & C. L. Cambiaso. 1975. A mechanism for the induction of immunological tolerance by antigen feeding: antigen-antibody complexes. J. Exp. Med. **142:** 1509–1519.
10. Hanson, D. G., N. M. Vaz, L. C. S. Maia, M. M. Hornbrook, J. M. Lynch & C. A. Roy. 1977. Inhibition of specific immune responses by feeding protein antigens. Int. Arch. Allergy Appl. Immunol. **55:** 526–532.
11. Swarbrick, E. T., C. R. Stokes & J. F. Soothill. 1979. Absorption of antigens after oral immunisation and the simultaneous induction of specific systemic tolerance. Gut **20:** 121–125.
12. Miller, S. D. & D. G. Hanson. 1979. Inhibition of specific immune responses by feeding protein antigens. IV. Evidence for tolerance and specific active suppression of cell mediated immune responses to ovalbumin. J. Immunol. **123:** 2344–2350.
13. Challacombe, S. J. & T. B. Tomasi. 1980. Systemic tolerance and secretory immunity after oral immunisation. J. Exp. Med. **152:** 1459–1472.
14. Titus, R. G. & J. M. Chiller. 1981. Orally induced tolerance: Definition at the cellular level. Int. Arch. Allergy Appl. Immunol. **65:** 323–328.
15. Askenase, P. W., B. J. Hayden & R. K. Gershon. 1975. Augmentation of delayed-type hypersensitivity by doses of cyclophosphamide which do not affect antibody responses. J. Exp. Med. **141:** 697–702.

16. MOWAT, A. MCI. & A. FERGUSON. 1981. Hypersensitivity reactions in the small intestine. 5. Induction of cell mediated immunity to a dietary antigen. Clin. Exp. Immunol. **43:** 574–582.
17. MOWAT, A. MCI., S. STROBEL, H. E. DRUMMOND & A. FERGUSON. 1982. Immunological responses to fed protein antigens in mice 1. Reversal of oral tolerance to ovalbumin by cyclophosphamide. Immunology **45:** 105–113.
18. MOWAT, A. MCI., A. FERGUSON. 1982. Migration inhibition of lymph node lymphocytes as an assay for regional cell mediated immunity in the intestinal lymphoid tissues of mice immunised orally with ovalbumin. Immunology. **47:** 365–370.
19. CLARKE, R. M. 1970. Mucosal architecture and epithelial cell production in the small intestine of the albino rat. J. Anat. **107:** 519–529.
20. FERGUSON, A. & D. MURRAY. 1971. Quantitation of intraepithelial lymphocytes in human jejunum. Gut **12:** 988–994.
21. MOWAT, A. MCI. 1981. Induction and expression of delayed hypersensitivity in the small intestine. PhD. Thesis, University of Edinburgh, Scotland.
22. ECKNAUER, R. & U. LOHRS. 1976. The effect of a single dose of Cyclophosphamide on the jejunum of specified pathogen-free and germ-free rats. Digestion **14:** 269–280.
23. SCHWARTZ, A., P. W. ASKENASE & R. K. GERSHON. 1978. Regulation of delayed-type hypersensitivity by cyclophosphamide sensitive T-cells. J. Immunol. **121:** 1573–1577.
24. NGAN, J. & L. S. KIND. 1978. Suppressor T cells for IgE and IgG in Peyer's patches of mice made tolerant by the oral administration of ovalbumin. J. Immunol. **120:** 861–865.
25. MATTINGLY, J. A. & B. H. WAKSMAN. 1978. Immunologic suppression after oral administration of antigen. I. Specific suppressor cells formed in rat Peyer's patches after oral administration of sheep erythrocytes, and their systemic migration. J. Immunol. **121:** 1878–1883.
26. GUY-GRAND, D., C. GRISCELLI & P. VASSALLI. 1978. The mouse gut T-lymphocyte, a novel type of T-cell: nature, origin and traffic in mice in normal and graft-versus-host conditions. J. Exp. Med. **148:** 1661–1677.

DISCUSSION OF THE PAPER

J. R. MCGHEE (*University of Alabama in Birmingham*): How do you know that a suppressor cell is involved in systemic tolerance *in vivo*? Could you summarize the CMI test that you used?

A. FERGUSON (*University of Edinburgh, Edinburgh, Scotland*): We transferred serum from animals fed with OVA, into the recipients, or sera from CY pretreated and OVA-fed donors. The abrogation of tolerance occurs if the recipients of the serum from OVA-fed animals are given cyclophosphamide. It is a CY-sensitive mechanism.

J. A. MATTINGLY (*Ohio State University, Columbus, Ohio*): Perhaps I have confirmed your results in an antibody system. Cyclophosphamide eliminated the suppressor cells from the spleen, but left the suppressor-inducer in the Peyer's patches. I was wondering whether the suppressor in the Peyer's patches can be a suppressor-effector for DTH rather than a suppressor-inducer for antibody.

J. M. PHILLIPS-QUAGLIATA (*New York University Medical Center, New York, N.Y.*): Have you tried to induce GVH reactions with peripheral node T-cells? If so, do they have the same effect on crypt length? Do mixed lymphocyte reaction (MLR) supernates have a similar effect?

FERGUSON: We have tried supernatants without any significant success. With regard to different cell types that induce GVH, we have used only spleen cells. Changes observed are really quite mild compared to those that have been reported by others.

DR. J. BIENENSTOCK (*McMaster University, Hamilton, Ontario, Canada*): What is the time scale of induction of mucosal CMI, and do you know whether your mesenteric lymph nodes contain functional suppressor cells? If so, when do they appear? Suppressor cells have been shown to appear first in Peyer's patches and then in mesenteric lymph nodes.

FERGUSON: Surprisingly, we found positive MIF tests from day 1 to day 21. When animals were refed, positive responses reappeared after 10 days. I am afraid we have no information on functional suppressor cells. Cells from Peyer's patches would be very difficult to use in the migration inhibition test.